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Tau Phosphorylation in Brain Slices: Pharmacological Evidence for Convergent Effects of Protein Phosphatases on Tau and Mitogen-Activated Protein Kinase

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SUMMARY

Tau is a neuron-specific, microtubule-associated protein that forms paired helical filaments (PHFs) of Alzheimer's disease when aberrantly phosphorylated. We have attempted to elucidate the protein kinases and phosphatases that regulate tau phosphorylation. Incubation of rat, human, and rhesus monkey temporal neocortex slices with the phosphatase inhibitor okadaic acid induced epitopes of tau similar to those found in PHFs. Okadaic acid (1–20 μM) induced variant forms of tau at 60–68 kDa, which were recognized by the monoclonal antibodies Alz-50 (in humans only) and 5E2 and two polyclonal antipeptide antisera, OK-1 and OK-2. The phosphorylation-sensitive monoclonal antibody Tau-1 failed to recognize the slowest mobility forms of tau after okadaic acid treatment. FK-520 (1–10 μM), a potent inhibitor of calcineurin activity, was tested in brain slices and found not to alter tau mobility. However, combinations of FK-520 (5 μM) and okadaic acid (100 nM) caused tau mobility shifts similar to those seen after 10 μM okadaic acid treatment; similar results were seen using the calcineurin-selective inhibitor cypermethrin. Treatment of human slices with 10 μM okadaic acid decreased both protein phosphatase 2A and calcineurin activity; FK-520 inhibited only protein phosphatase 2B activity. A proposed tau-directed kinase, 42-kDa mitogen-activated protein kinase ($p42^{mapk}$), was activated by okadaic acid (>100 nM) but not FK-520 (5 μM). Nerve growth factor (100 ng/ml) activated $p42^{mapk}$, particularly when used in combination with 100 nM okadaic acid; changes in tau mobility were seen when this kinase was activated. Forskolin (2 μM) antagonized the effects of nerve growth factor on both $p42^{mapk}$ activity and tau phosphorylation; forskolin alone had little effect on PHF-like tau formation induced by phosphatase inhibitors. These results outline complex interactions between tau-directed protein kinases and protein phosphatases and suggest potential sites for therapeutic intervention.

Tau is a neuron-specific, heat-stable, microtubule-associated protein (48–68 kDa). Multiple isoforms are generated from a single gene through alternative mRNA splicing, and each isoform can be phosphorylated (1). Because highly phosphorylated tau protein is the primary constituent of the PHFs of Alzheimer's disease, considerable efforts have been directed towards elucidation of the protein kinases and phosphatases that regulate tau phosphorylation. *In vitro* studies indicate that tau is phosphorylated by cAMP-dependent protein kinase, calmodulin kinase II, and protein kinase C (2–5). Recent studies have focused on the role of proline-directed protein kinases (e.g., $p42^{mapk}$ and glycogen synthase kinase-3), cdk2, and cdk5 in the regulation of tau phosphorylation and microtubule binding (6–12).

Dephosphorylation of tau is likely to play a key role in the regulation of tau; both PP2A and calcineurin (PP2B) dephosphorylate tau *in vitro* (13–16). Incubation of human brain slices or cultured rat cortical neurons with okadaic acid leads to accumulation of phosphorylated forms of tau (17–19). However, more work is needed to determine which phosphatases dephosphorylate tau *in vivo*.

Specific sites of tau phosphorylation have been associated with altered tau function and formation of PHFs. In PHF preparations (i.e., A68) from Alzheimer brain, these variably

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ABBREVIATIONS: PHF, paired helical filament; cdk, cyclin-dependent kinase; $p42^{mapk}$, 42-kDa mitogen-activated protein kinase; MAPK, mitogen-activated protein kinase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophopresis; A68, 68-kDa antigen; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitroblue tetrazolium; FKBP, FK-506-binding protein; DMSO, dimethylsulfoxide; NGF, nerve growth factor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PP1 and PP2, protein phosphatases 1 and 2.

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include (using the numbering system of the longest human isoform of tau, ht-411) Ser-46, Thr-191, Ser-202, Thr-231, Ser-235, Ser-262, Ser-396 and a site between residue 191 and residue 225 (20–22). In particular, phosphorylation of Ser-262 has been shown to alter the microtubule-binding activity of tau (23). Ser-202 and Ser-396 are phosphorylated in fetal tau, suggesting that tau phosphorylation in Alzheimer brain recapitulates that seen in early development. In rats, fetal tau is correspondingly phosphorylated at up to 10 sites, including Thr-172, Ser-189, Ser-190, Ser-193, Ser-208, Thr-222, Ser-226, Ser-387, Ser-391, and Ser-395; in adult rats, sites include Thr-172, Ser-190, Ser-193, Thr-222, and Ser-395 (24). The majority of these sites are Ser/Thr-Pro sites, suggesting that proline-directed protein kinases actively phosphorylate tau and that such activity is present in fetal brain. Alternatively, tau-directed protein phosphatases may be inactive during early development (25).

Polyclonal and monoclonal antibodies that recognize specific epitopes of tau have been developed. Epitope mapping studies have shown that monoclonal antibody Tau-1 recognizes a phosphorylation-sensitive (or conformation-sensitive) epitope between residue 189 and residue 207, whereas monoclonal antibody 5E2 detects an epitope between residue 156 and residue 175 (26). Alz-50, a monoclonal antibody prepared against brain homogenates from patients with Alzheimer's disease, recognizes Alzheimer-specific A68 concentrated in neurofibrillary tangles; its epitope is likely to be in the extreme amino terminus of tau (27). Each is useful for delineation of specific patterns of tau phosphorylation; Tau-1 fails to recognize the most phosphorylated forms of tau, but 5E2 recognizes most major forms of tau in multiple species, including the antigenic peptide A68. The human-specific Alz-50 is useful for detection of highly phosphorylated forms of human tau, which are characteristically seen as a triplet of peptides between 60 and 68 kDa, although it more weakly detects nonphosphorylated isoforms as well. We have generated two polyclonal antipeptide antisera, the amino terminus-specific OK-1 and the carboxyl terminus-specific OK-2, that detect tau isoforms independently of their state of phosphorylation, which is similar to the detection profile of monoclonal antibody 5E2.

The kinase $p42^{mapk}$ has been reported to induce tau phosphorylation (9). This enzyme is activated by phosphorylation at Tyr-185 and Thr-183 by MAPK kinase (28). Thr-183 is dephosphorylated by PP2A, which suggests a point of convergence between the protein kinases and protein phosphatases that alter tau phosphorylation. $p42^{mapk}$ can be activated via signaling from tyrosine kinase growth factor receptors. NGF may activate this pathway, eventually leading to $p42^{mapk}$ activation (29). Growth factor activation of MAPK isoforms can be down-regulated by cAMP-dependent protein kinase, which is thought to act on Raf-1 (30). This negative regulation provides for receptor cross-talk between tyrosine kinase growth factor receptors and receptors coupled to adenylate cyclase. Thus, it is possible that *in vivo* phosphorylation of tau protein and the establishment of an equilibrium between phosphorylation and dephosphorylation may involve regulation of $p42^{mapk}$ via multiple and convergent pathways.

In this study, we used brain slices from rat, human, and rhesus monkey to elucidate complex interactions between the enzymes that control tau phosphorylation. We now report that the phosphatase inhibitor okadaic acid (at concentrations of 1–10 μM) or drug combinations (5 μM FK-520 or 100 nM cypermethrin plus 100 nM okadaic acid) that inhibited both calcineurin (as determined by enzyme activity assays) and PP2A induced PHF-like tau, as determined by electrophoretic mobility shifts detected with multiple antibodies. In addition, okadaic acid inhibition of PP2A may concomitantly activate $p42^{mapk}$, leading to tau-directed protein phosphorylation at PHF-like sites. NGF, in combination with okadaic acid (≥100 nM) or FK-520 (5 μM), was able to induce PHF-like tau and activate $p42^{mapk}$; this effect was blocked by forskolin treatment. These effects are likely to be mediated by convergent effects of phosphatases; phosphatase inhibitors block direct dephosphorylation of tau by PP2A and calcineurin while causing simultaneous activation of $p42^{mapk}$, which can then phosphorylate tau at Ser/Thr-Pro sites.

Experimental Procedures

Preparation and treatment of human tissue slices. A specimen of human lateral temporal neocortex (1 cm^3), routinely resected in the course of excision of an epileptogenic focus in the hippocampus, was used as a source for tissue slices in human studies. Portions of the excised temporal neocortex were also prepared for routine histology and found to be normal in appearance. Patients undergoing elective epilepsy surgery signed informed consent forms regarding disposition of this tissue from surgery; this experimental protocol was approved by the Clinical Investigation Committee at the Milton S. Hershey Medical Center (Protocol EP-515).

Immediately after excision, neocortex was immersed in ice-cold preincubation buffer (10 mM HEPES, pH 7.4, containing 125 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 15 mM $MgSO_4$, and 10 mM glucose; oxygenated with 95% O_2/5% CO_2); this buffer was designed to keep Ca^{2+} levels minimal (to prevent Ca^{2+}-mediated enzyme activities) and to include high levels of Mg^{2+} (to minimize ischemic damage to the tissue) (18). Sections (225 μm) were prepared with a Sorvall tissue chopper (DuPont, Wilmington, DE), randomized into groups of six to eight slices, and incubated (30 min, at 37°) in oxygenated preincubation buffer. This solution was removed and replaced with labeling buffer (10 mM HEPES, pH 7.4, containing 125 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 5 mM $MgSO_4$, and 1.5 mM $CaCl_2$), usually containing 250 μCi of $^{32}P_i$ (>3000 Ci/mmol, carrier free; Amersham). After 45 min in labeling buffer, slices were treated for various lengths of time with one or more of the following: okadaic acid (100 nM to 20 μM; GIBCO-BRL) or the FK-506 analog FK-520 (1–10 μM; a generous gift from Dr. Nolan Sigal, Merck, Rahway, NJ). Both drugs were dissolved as concentrated stocks in DMSO; this vehicle was included in control incubations (0.1%) and was without effect on any of the measured variables.

Reactions were terminated by removal of labeling buffer and addition of either 200 μl of a solution containing 2% SDS, 25 mM K_2HPO_4, 2 mM EGTA, 10 mM EDTA, and 50 mM NaF or, for samples to be used in phosphatase assays, 200 μl of 50 mM MOPS, pH 7.0, containing 2 mM $Mg(C_2H_3O_2)_2$, 2 mM $Mn(C_2H_3O_2)_2$, 15 mM β-mercaptoethanol, 2 mM $CaCl_2$, and a protease inhibitor mixture containing leupeptin (100 μM), soybean trypsin inhibitor (100 μg/ml), aprotinin (100 μM), and phenylmethylsulfonyl fluoride (100 μM). Tissues were then disrupted by sonication. Protein was measured using the bicinchoninic acid reagent (Pierce Chemical Co.) according to the manufacturer's instructions, 20 μl of 0.1 M dithiothreitol were then added to the rest of each sample, and the samples were stored at −20°. Samples were heated at 90° for 5 min and centrifuged for 2 min at 13,000 × *g* before electrophoresis.

Preparation and treatment of rat brain slices. Rat temporal lobe was obtained after decapitation of male Sprague-Dawley rats. The temporal lobe was removed using a brain mold (Activational

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Systems, Warren, MI), with care being taken to eliminate residual hippocampus. Sections of temporal cortex or hippocampus (225 μm) were prepared using a Sorvall tissue chopper (Du Pont). Slices were immersed in ice-cold preincubation buffer. Slices were randomized (six to eight slices/group) and incubated (30 min, at 37°) in oxygenated preincubation buffer. Other treatments were as described for human brain slices. After the 45-min labeling period, slices were treated for various lengths of time with one or more of the following agents: okadaic acid (100 nM to 10 μM) the FK-506 analog FK-520 (1–20 μM), the selective calcineurin inhibitor cypermethrin (100 nM; LC Laboratories), NGF (100 ng/ml; Boehringer Mannheim), and forskolin (2 μM; Sigma). All drugs except NGF were dissolved as concentrated stock solutions in DMSO; this vehicle was included in control incubations (0.1%) and had no effect on any of the measured variables. NGF was dissolved in sterile phosphate-buffered saline containing 1 mg/ml bovine serum albumin. Reactions were terminated by removal of labeling buffer and addition of one of three buffers, i.e., 200 μl of a NaF-SDS stop solution as described for human slice experiments, 200 μl of a lysis buffer (for $p42^{mapk}$ assays) containing 50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM Na_3VO_4, 100 mM NaF, 10 mM EDTA, 10 mM EGTA, and a protease inhibitor cocktail containing leupeptin (100 μM), soybean trypsin inhibitor (100 μg/ml), aprotinin (100 μM), and phenylmethylsulfonyl fluoride (100 μM), or 200 μl of the phosphatase assay buffer previously described for human slices. After sonication to disrupt the tissue, protein was measured using the bicinchoninic acid reagent; 20 μl of 0.1 M dithiothreitol was added to the rest of each sample (except samples to be immunoprecipitated), and samples were stored at −20°. Before electrophoretic analysis, samples were heated at 90° for 10 min and then centrifuged at 13,000 × *g* for 5 min.

Rhesus monkey brain removal and slice preparation. The population of adult male monkeys used in these experiments had undergone a series of procedures in previous studies and were euthanized (100 mg/kg sodium pentobarbital, intravenously) after an opthalamic procedure and immediately necropsied. Brains were rapidly removed, with an average interval of 4–6 min from cessation of the heartbeat to slice preparation. Temporal lobe slices were prepared as described above for human and rat temporal lobe samples. Rhesus monkey brains were a generous gift from Drs. C. M. Lang and M. Hardigan (Department of Comparative Medicine, Pennsylvania State University College of Medicine).

Preparation of OK-1 and OK-2 polyclonal antisera. The following peptides were synthesized using a Milligen 9500 peptide synthesizer. Peptide OK-1 (NH-MAEPRQEFEVMEDHAGTYGLGC-COOH) corresponded to amino acids 1–21 of human tau; peptide OK-2 (NH-PQLATLADEVSASLAKQGLC-COOH) corresponded to amino acids 334–352 of human tau. Both were synthesized using 9-fluorenylmethoxycarbonyl chemistry, using a cysteine carboxyl-terminal resin. Peptides were coupled to keyhole limpet hemocyanin (Sigma) via a cysteine linkage, using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (Pierce) as a cross-linking agent. After coupling, peptides were desalted on Biogel P-2 (Bio-Rad) and stored at −80° until use. New Zealand White rabbits were used as hosts; injection protocols involved an initial inoculation of 1.0 mg of conjugate (multiple intradermal and intraperitoneal sites), followed by boosts with 250–500 μg of antigen at 3-week intervals. Antisera were monitored for immunoreactivity using immunoblot analysis of brain proteins.

After immunoreactivity was established on immunoblots, antisera were purified using ammonium sulfate precipitation (50%) and subsequent affinity purification with the appropriate peptide conjugate coupled to CNBr-activated Sepharose 4B (Pharmacia), using conventional elution strategies (0.2 M glycine buffer, pH 2.4). Affinity-purified antisera were stored in 40% glycerol and kept at 4°. Both peptide antisera recognized tau from human, rat, and rhesus monkey brain.

Tau-1 monoclonal antibody recognizes a phosphorylation-sensitive epitope and was purchased from Boehringer-Mannheim. Alz-50 monoclonal antibody was a generous gift from Dr. Peter Davies (Albert Einstein College of Medicine) and 5E2 monoclonal antibody was a generous gift from Dr. Kenneth Kosik (Harvard University). Polyclonal anti-rat MAPK-R2 antibody was purchased from Upstate Biotechnology, Inc.

Preparation of phosphorylated R_{II} peptide. The peptide R_{II} (bovine cardiac cAMP-dependent protein kinase regulatory subunit amino acids 81–99, NH-DLDVPIPGRFDRRVSVCAE-COOH) was synthesized using a Milligen 9500 peptide synthesizer with 9-fluorenylmethoxycarbonyl chemistry. After synthesis and purification, R_{II} was phosphorylated using the catalytic subunit of cAMP kinase, and free radioactivity was removed as described previously (31).

Calcineurin activity assay. Dephosphorylation of phosphorylated R_{II} peptide was used to determine changes in calcineurin activity, as described previously (31). Brain slices were treated with phosphatase inhibitors as described in the previous section. Slices were homogenized in 50 mM Tris·HCl, pH 7.4, containing protease inhibitors, and 100 μg of protein were used for each reaction. Calmodulin and Ca^{2+} were added to each reaction and incubated for 5 min at 30°. Reactions were terminated by addition of 10% trichloroacetic acid and 0.5 mg/ml bovine serum albumin. After centrifugation for 10 min at 13,000 × *g*, ^{32}P released in the supernatant was quantitated by liquid scintillation counting. Interassay variability was determined by running three duplicate assays of the same treatment groups in three different rats, whereas intra-assay variability was determined by running three duplicate assays of the same treatment groups from the same animal.

Activity measurements were also performed using purified bovine brain calcineurin (a gift of Dr. Randall Kincaid, Penn State University); reactions contained 1 μg of calmodulin, 1 mM Ca^{2+}, and 1 mM $MgSO_4$, as described previously (18, 31). Control reactions lacked either enzyme, calmodulin, or Ca^{2+}.

PP2A activity assay. Myelin basic protein was phosphorylated as described (32) and used as a substrate for PP2A. Assays of brain slice extracts were carried out as described (32), in a reaction containing 50 mM Tris·HCl, pH 7.0, 10% glycerol, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 14 mM β-mercaptoethanol, 0.1 mg/ml bovine serum albumin, 0.03% Brij-35, 1 mM EDTA, and 100,000 cpm of ^{32}P-labeled myelin basic protein. After 10 min at 30°, 10% trichloroacetic acid was added to stop the reaction, and the reaction mixture was centrifuged for 2 min at 13,000 × *g*. Supernatant was used for assay of released ^{32}P from myelin basic protein.

SDS-PAGE and immunoblotting procedures. Samples from human brain prepared as described above were subjected to one-dimensional, 10% SDS-PAGE and transferred to nitrocellulose membranes (18). In some experiments, samples were subjected to two-dimensional SDS-PAGE, followed by transfer to nitrocellulose membranes. Nonspecific binding sites on the membrane were blocked by incubation of the blots with blocking buffer (Tris-buffered saline containing 5% nonfat dry milk). Blots were then incubated with one of the following primary monoclonal antibodies diluted in blocking buffer: Alz-50 (1/5 dilution), 5E2 (1/40 dilution), or Tau-1 (1 μg/ml). Two preparations of affinity-purified rabbit polyclonal anti-peptide antisera against tau (OK-1 and OK-2) were diluted 1/500 in blocking buffer. For monoclonal antibodies (Alz-50, Tau-1, and 5E2), immune complexes were detected using alkaline phosphatase-conjugated rabbit anti-mouse secondary antibody (diluted 1/1000 in blocking buffer; Jackson Immunoresearch); affinity-purified rabbit polyclonal antisera (OK-1, OK-2, and R2) were detected using alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (diluted 1/1000 in blocking buffer). Both preparations were visualized with the BCIP/NBT chromogen system, as described (33).

Immunoprecipitation of $p42^{mapk}$. Brain homogenates (250 μg of total protein) sonicated in the previously defined lysis buffer were subjected to immunoprecipation using a 4-hr incubation (4°) with anti-rat MAPK-R2 polyclonal antiserum (1 μg/250 μg of homogenate). Immune complexes were isolated by addition of 50 μl of Protein A-Sepharose beads (Pharmacia), which were suspended in a bead wash buffer containing Tris·HCl, pH 8.0, 100 mM NaCl, 0.5%

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Nonidet P-40, 2 mM Na_3VO_4, 100 mM NaF, 10 mM EDTA, 10 mM EGTA, and the protease inhibitors mentioned previously for the lysis buffer. After incubation for 1 hr at 4°, samples were centrifuged at 13,000 × *g* at 4° for 5 min, and the pellet was washed five times with the bead wash buffer.

$p42^{mapk}$ activity assay. After the last bead wash, samples were used in an enzyme assay designed to measure $p42^{mapk}$ activity. After the last wash was removed from the beads, the following was added to each pellet (on ice): 5 μl of assay buffer stock solution (containing 250 mM Tris, pH 7.0, 125 mM $MgCl_2$, and 0.25 mM Na_3VO_4), 10 μl of dilution buffer (containing 250 mM HEPES, pH 7.0, 10% glycerol, and 0.1% Nonidet P-40), 5 μl of a 1.5 mM stock solution of a $p42^{mapk}$ substrate (amino acids 95–98 of myelin basic protein; UBI), and 5 μl of 0.5 mM ATP stock solution (containing [γ-^{32}P]ATP; specific activity, 1500 cpm/pmol), which began the reaction. The kinase subtrate was resuspended in assay buffer stock solution. Upon addition of the ATP stock solution, samples were incubated for 15 min at 30°, with gentle mixing of samples while immune complex beads were prevented from adhering to the side of the tube. Reactions were terminated by placing the samples directly on ice. Five microliters of each sample were then spotted on a cellulose-coated, thin layer chromotagraphy plate (20 cm × 20 cm, 0.1 mm thick; Alltech Associates, Deerfield, IL). The thin layer chromatography plates were developed using a Hunter, thin layer, peptide-mapping system (model HT-7000; CBS Scientific, Delmar, CA) for 30 min at 1500 V. The extent of peptide phosphorylation was determined by subjecting the plates to radiometric scanning using a Betagen radiometric scanner. The picomoles of $^{32}P_i$ incorporated into the peptide as a result of $p42^{mapk}$ activity were determined for each group after Betagen scanning of the separated phosphorylated peptide.

PhosphorImager analysis. After immunoprecipitation and washing, 60 μl of a standard SDS stop solution were added to a few selected groups and samples were heated for 10 min at 90°. Samples were then centrifuged at 13,000 × *g* for 5 min, and the supernatant was used for SDS-PAGE analysis. Resolved isoforms were transferred to nitrocellulose as detailed above. Blots were scanned on a Molecular Dynamics model 420E PhosphorImager to determine $^{32}P_i$ incorporation. In several experiments, transferred proteins were subjected to immunoblotting to verify successful immunoprecipitation and transfer.

Results

Control by multiple protein phosphatases of tau phosphorylation in rat brain. Previous experiments from our laboratory (18) indicated that okadaic acid, at concentrations greater than 5 μM, could shift tau electrophoretic mobility to a value similar to that of PHF preparations from Alzheimer brain (e.g., A68 proteins). Fig. 1A shows the concentration-dependent effects of okadaic acid on immunoreactive tau mobility (OK-1 antiserum detection) in metabolically active, rat temporal neocortex slices. The characteristic shift in tau mobility, including the appearance of a new peptide at 68 kDa, occurred at concentrations of okadaic acid of ≥5 μM. Because okadaic acid potently inhibits PP1 and PP2A at nanomolar levels *in vitro* (PP1, IC_{50} = 20 nM; PP2A, IC_{50} = 0.2 nM) but does not inhibit calcineurin until micromolar concentrations are reached (IC_{50} = 5 μM), these results suggest that calcineurin plays a role in controlling the state of tau phosphorylation.

To determine whether calcineurin was the primary tau-directed phosphatase in rat temporal neocortex, rat brain slices were incubated with the calcineurin-specific inhibitor FK-520. As seen in Fig. 1B, concentrations of FK-520 ranging from 1 to 10 μM failed to induce significant mobility shifts in tau isoforms. This indicated that, although calcineurin plays

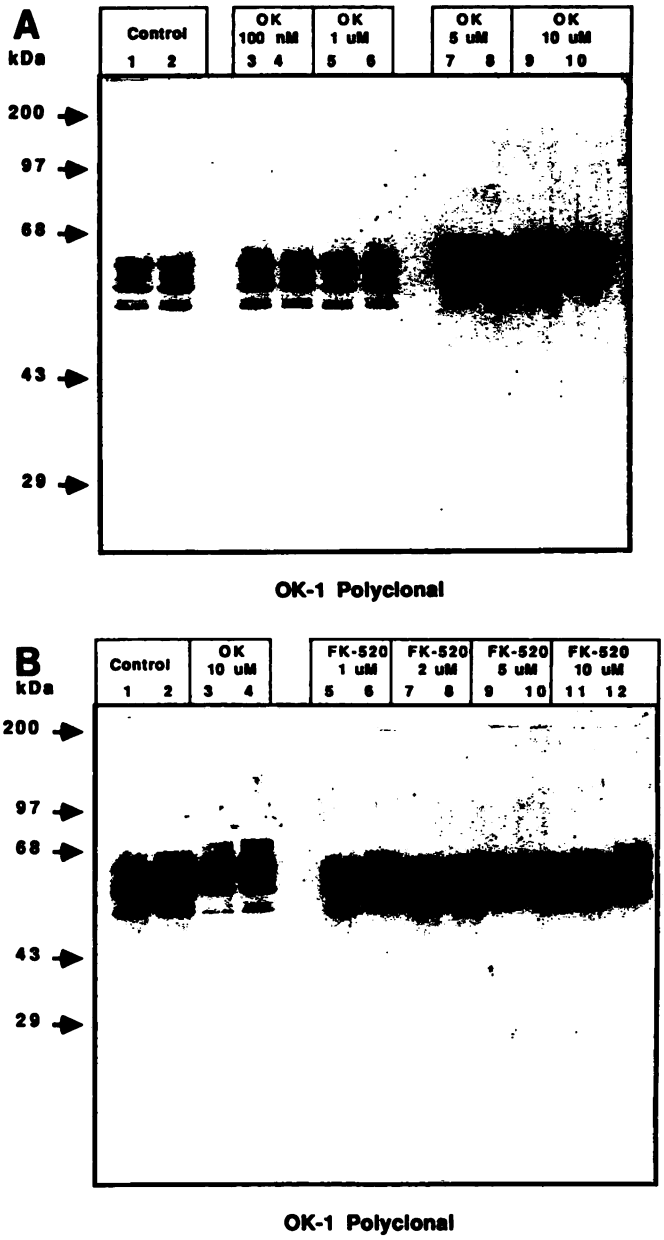

Fig. 1. Okadaic acid induction of concentration-related changes in tau immunoreactivity and mobility. A, Slices of Sprague-Dawley rat temporal neocortex were prepared and treated as described in Experimental Procedures. Samples (100 μg of protein) were subjected to 10% SDS-PAGE, and resolved proteins were transferred to nitrocellulose membranes and incubated with the OK-1 polyclonal antiserum. Immune complexes were visualized using an alkaline phosphatase-conjugated secondary antibody, using the BCIP/NBT chromogen system. *Lanes 1* and *2*, independent samples incubated with DMSO vehicle (control); *lanes 3-10*, samples showing effects of increasing concentrations of okadaic acid (*OK*) on the electrophoretic mobility of immunoreactive tau protein from rat temporal lobe. B, Slices of rat temporal lobe were prepared and treated as described in Experimental Procedures. Samples (100 μg of protein) were electrophoresed as described and incubated with the OK-1 polyclonal antiserum. *Lanes 1* and *2*, independent samples incubated with DMSO vehicle (control); *lanes 3* and *4*, samples showing the effects of 10 μM okadaic acid on tau mobility; *lanes 5-12*, samples showing the lack of effect of increasing concentrations of FK-520 on the electrophoretic mobility of immunoreactive tau protein from rat temporal lobe.

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some role in tau dephosphorylation, inhibiting this phosphatase alone does not yield upward shifts in tau mobility similar to that seen with okadaic acid alone. Similar results were seen in the immunoreactive pattern of tau from rat hippocampal slices that had been treated with FK-520 (data provided to reviewers), suggesting that this effect is not brain region specific.

Because FK-520 alone failed to induce PHF-like tau, we reasoned that inhibition of multiple phosphatases was necessary to induce mobility shifts in tau. Rat temporal lobe slices were incubated under conditions that favored differential inhibition of PP1 and PP2A (with 100 nM okadaic acid), calcineurin (with 5 μM FK-520), or both PP2A and calcineurin (with 10 μM okadaic acid or 100 nM okadaic acid plus 5 μM FK-520). Fig. 2A, *lanes 2-5* (an OK-1 immunoblot), shows that okadaic acid alone produced PHF-like tau shifts at micromolar levels. When present alone, FK-520 (5 μM) had no effect on tau mobility, as noted previously. However, when FK-520 (5 μM) and okadaic acid (100 nM) were used in combination, significant shifts in tau mobility were seen. This suggests that both calcineurin and PP2A (and possibly PP1) play significant roles in controlling phosphorylation of tau. Fig. 2B demonstrates that the Tau-1 monoclonal antibody, although able to recognize slight upward mobility shifts in the lower molecular mass isoforms, did not recognize the highest molecular mass peptide detected by OK-1 in Fig. 2A, *lanes 2* and *5*.

Alteration by phosphatase inhibitors of tau phosphorylation in primates. Incubation of human brain slices with okadaic acid (5–20 μM) caused marked slowing of tau mobility, with the appearance of a peptide at 68 kDa and variable disappearance of tau peptides at faster mobilities (43–55 kDa). Fig. 3A shows data for one representative patient (of a total of 24) treated with increasing concentrations of okadaic acid. Monoclonal antibodies Alz-50 and 5E2 were used to show that peptides of 60–68 kDa appeared as a result of okadaic acid treatment; as expected, the highest mobility peptide at 68 kDa was much more weakly detected by Tau-1 monoclonal antibody. These data suggested that okadaic acid led to increased phosphorylation of tau protein through phosphatase inhibition. To confirm the involvement of multiple phosphatases, temporal lobe slices from another patient were incubated with okadaic acid and FK-520 alone and in combination, followed by detection of tau with Alz-50 monoclonal antibody. As shown in Fig. 3B, the combination of okadaic acid (100 nM) and FK-520 (5 μM) was able to induce PHF-like tau to an extent similar to that seen with micromolar concentrations of okadaic acid. As seen in rats, FK-520 alone did not induce PHF-like tau. Three additional patients were used to confirm this finding, using different antibody preparations (OK-1, OK-2, and 5E2) (data not shown). Similar results were seen in rhesus monkey temporal lobe slices; as shown in Fig. 3C, affinity-purified OK-1 antiserum was able to detect tau in rhesus monkey brain. Neither FK-520 nor another

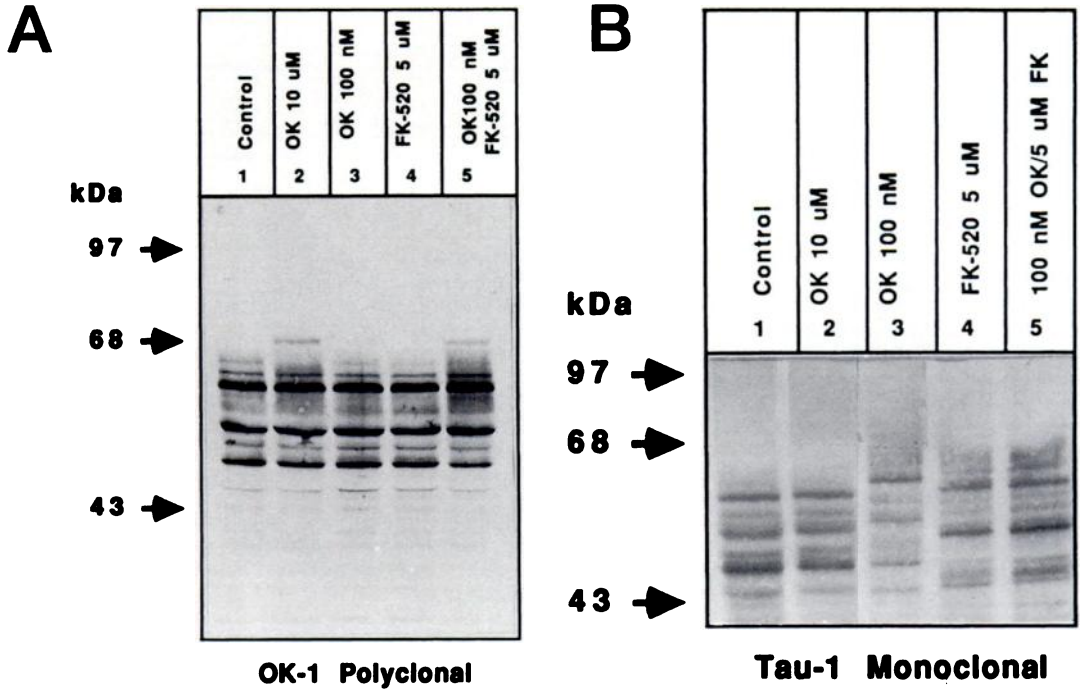

Fig. 2. Effects of FK-520 and okadaic acid on tau phosphorylation in rat temporal lobe slices. A, Slices of rat temporal lobe were prepared and treated as described in Experimental Procedures. Samples (100 μg of protein) were electrophoresed as described and incubated with the OK-1 polyclonal antiserum. *Lane 1*, independent sample incubated with DMSO vehicle (control); *lane 2*, sample showing the effects of 10 μM okadaic acid (*OK*) on the development of PHF-like tau in rat temporal lobe slices; *lane 3*, sample showing the minimal effects of 100 nM okadaic acid on tau mobility; *lane 4*, sample showing the lack of effect of 5 μM FK-520 alone on increased tau mobility; *lane 5*, sample showing the effect of a combination of 100 nM okadaic acid and 5 μM FK-520 on PHF-like tau development. B, Slices of rat temporal lobe were prepared and treated as stated previously. Samples (100 μg) were electrophoresed as described and incubated with the Tau-1 monoclonal antibody. *Lane 1*, independent sample incubated with DMSO vehicle (control); *lane 2*, sample showing that Tau-1 is unable to recognize the highest molecular mass peptide, as had been detected in A; *lane 3*, sample showing the lack of a significant effect of 100 nM okadaic acid on tau mobility; *lane 4*, sample showing the relative inability of FK-520 to alter tau mobility; *lane 5*, sample showing the inability of Tau-1 monoclonal antibody to recognize the highest molecular mass peptide, as had been seen in A.

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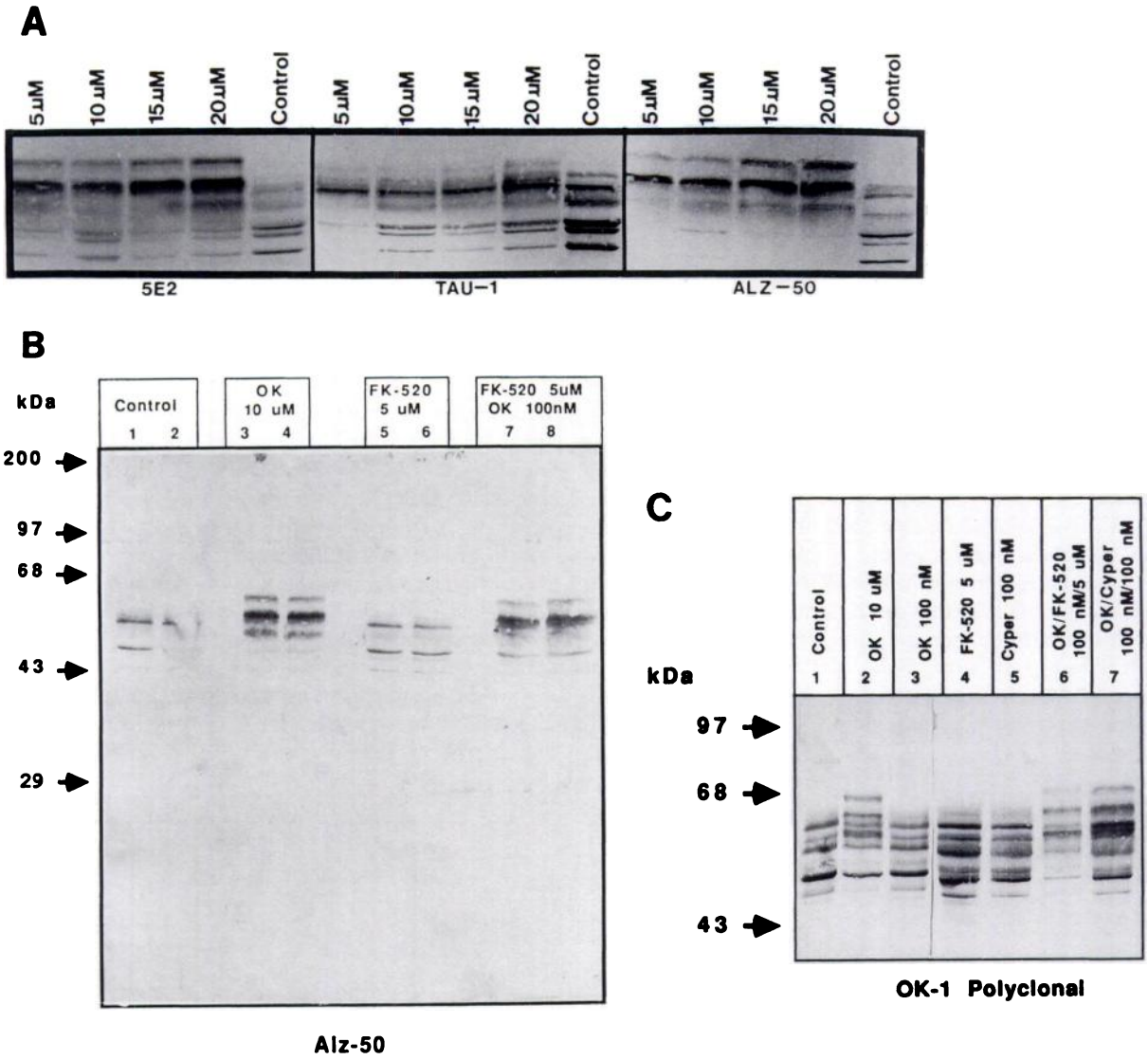

Fig. 3. Effects of FK-520 and okadaic acid on tau phosphorylation in primate temporal lobe slices. A, Metabolically active human temporal lobe slices were treated with okadaic acid (5–20 μM) and subjected to 10% SDS-PAGE and immunoblotting, with visualization via the BCIP/NBT chromogen system after incubation with alkaline phosphatase-conjugated secondary antibodies. Each section represents a replica blot incubated with monoclonal antibody Alz-50, 5E2, or Tau-1. Tau-1 antibody failed to recognize the lowest mobility form of tau at 68 kDa. A total of four to six slices from a single patient were used for this experiment. B, Sections of human temporal lobe were obtained and treated as described in Experimental Procedures. Samples (100 μg) were subjected to 10% SDS-PAGE, transferred to nitrocellulose membranes, and incubated with the human tau-specific monoclonal antibody Alz-50. *Lanes 1* and *2*, independent samples incubated with DMSO vehicle (control); *lanes 3* and *4*, samples showing the effects of 10 μM okadaic acid (*OK*) on the development of PHF-like tau in human temporal lobe slices; *lanes 5* and *6*, samples showing the lack of effect of 5 μM FK-520 alone on hyperphosphorylated tau development; *lanes 7* and *8*, samples showing the effects of 100 nM okadaic acid plus 5 μM FK-520 on the development of PHF-like tau in human temporal lobe slices. C, Slices of rhesus monkey temporal lobe were prepared and treated as described in Experimental Procedures. Samples (100 μg of protein) were electrophoresed as described and incubated with the OK-1 polyclonal antiserum. *Lane 1*, independent sample incubated with DMSO vehicle (control); *lane 2*, sample showing the effects of 10 μM okadaic acid on the development of PHF-like tau in rhesus monkey temporal lobe slices; *lane 3*, sample showing the lack of effect of 100 nM okadaic acid on PHF-like tau development; *lane 4*, sample showing the lack of effect of 5 μM FK-520 on electrophoretic tau mobility; *lane 5*, sample showing the lack of effect of 100 nM cypermethrin alone on tau mobility; *lane 6*, sample demonstrating the combination effect of 5 μM FK-520 and 100 nM okadaic acid on PHF-like tau development; *lane 7*, sample demonstrating a similar combination effect of 100 nM cypermethrin and 5 μM FK-520 on the development of highly phosphorylated tau in rhesus monkey temporal lobe slices.

calcineurin-selective inhibitor, cypermethrin, alone could yield PHF-like shifts in tau mobility. However, when either drug was used in combination with 100 nM okadaic acid, which alone also produced no visible shifts in tau mobility, the characteristic upward shift in the immunoreactive pattern of tau was seen. The inactive analog FK-818 had no effect on tau mobility, either alone or in combination with okadaic acid (data not shown).

Effects of NGF and forskolin on tau phosphorylation. The potential tau-directed kinase p42^{mapk} can be normally activated via a cascade of phosphorylation events that are triggered by extracellular factors (e.g., NGF) acting via tyrosine kinase receptors (e.g., NGF receptor). Because NGF can activate p42^{mapk}, rat temporal lobe slices were incubated with NGF alone and in combination with okadaic acid or FK-520. As shown in Fig. 4, NGF alone produced minimal

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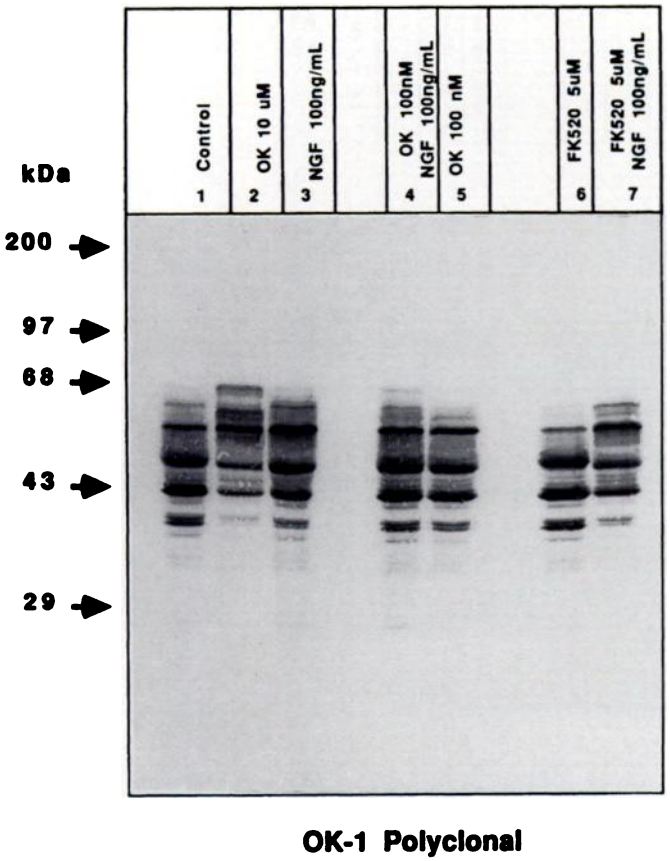

Fig. 4. Effects of NGF, FK-520, and okadaic acid on tau phosphorylation in rat temporal lobe slices. Slices of rat temporal lobe were prepared and treated as described in Experimental Procedures; resolved proteins (100 μg) were transferred to nitrocellulose and incubated with OK-1 polyclonal antiserum. *Lane 1*, independent sample incubated with DMSO vehicle (control); *lane 2*, sample showing the effects of 10 μM okadaic acid (*OK*) on the development of PHF-like tau in rat temporal lobe slices; *lane 3*, sample showing the effect of NGF (100 ng/ml) alone on tau mobility; *lane 4*, sample showing the effect of 100 nM okadaic acid plus NGF (100 ng/ml) on PHF-like tau development; *lane 5*, sample demonstrating the lack of effect of 100 nM okadaic acid on PHF-like tau development; *lane 6*, sample showing the lack of effect of 5 μM FK-520 alone on tau mobility; *lane 7*, sample showing the development of PHF-like tau after a combination treatment with 5 μM FK-520 and NGF (100 ng/ml).

shifts in tau mobility. However, when NGF was used in combination with 100 nM okadaic acid or 5 μM FK-520, PHF-like tau formation was observed, with the appearance of a 68-kDa peptide.

Forskolin-induced activation of cAMP-dependent protein kinase can inhibit the activation of $p42^{mapk}$ via inactivation of Raf-1. As shown in Fig. 5, the effects of combination treatment with NGF (100 ng/ml) and 100 nM okadaic acid on PHF-like tau development were antagonized by forskolin; both amino terminus-directed OK-1 (Fig. 5A) and carboxyl terminus-directed OK-2 (Fig. 5B) antisera were used to document mobility shifts in tau. Tau-1 monoclonal (Fig. 5C), however, failed to recognize the lowest mobility isoforms that had been detected in Fig. 5, A and B, *lanes 2* (10 μM okadaic acid) and *lanes 6* (100 ng/ml NGF plus 100 nM okadaic acid). Forskolin alone, however, had no effect on the patterns of PHF-like tau generated solely by phosphatase inhibitors (Fig. 5D).

Effects of phosphatase inhibitors and NGF on calcineurin activity. Changes in tau phosphorylation were correlated with alterations in calcineurin activity, as determined using dephosphorylation of ^{32}P-labeled R_{II} peptide after specific pharmacological treatments in slice preparations. Fig. 6A demonstrates that incubation of rat temporal lobe slices with either 10 μM okadaic acid or 5 μM FK-520 led to a decrease in calcineurin activity. Nanomolar levels of okadaic acid produced no changes in PP2B activity, compared with control. Fig. 6B indicates that incubation of rat temporal lobe slices with NGF and/or forskolin did not alter calcineurin activity; however, FK-520 blocked calcineurin activity. Fig. 6C is a control *in vitro* phosphatase assay using purified calcineurin; removal of Ca^{2+} or calmodulin blocked activity. Okadaic acid (10 μM) strongly inhibited calcineurin activity; however, incubation with 5 μM FK-520 alone did not inhibit activity due to the absence of the obligate mediators of inhibition, the FKBPs.

Effects of phosphatase inhibitors on PP2A and calcienurin activity in human temporal lobe slices. Both PP2A and PP2B activities were measured in human temporal lobe slices. Fig. 7B shows that incubation of human temporal lobe slices with either 10 μM okadaic acid or 5 μM FK-520 led to a significant decrease in PP2B activity in human brain homogenates. Fig. 7A demonstates the effects of phosphatase inhibitors on PP2A activity, using phosphorylated myelin basic protein as the substrate. Incubation with 100 nM okadaic acid decreased PP2A activity by 30%, whereas 10 μM okadaic acid decreased PP2A by 50%. Thus, 10 μM okadaic acid inhibited both PP2A and calcineurin, whereas FK-520 inhibited only calcineurin.

Effects of phosphatase inhibitors and NGF on $p42^{mapk}$ activity. To directly determine whether $p42^{mapk}$ was activated by phosphatase inhibitors and directly phosphorylated tau, this kinase was immunoprecipitated from each treatment group and used to phosphorylate a synthetic peptide substrate (myelin basic protein). Fig. 8A shows that okadaic acid, at concentrations of 100 nM and greater, induced similar increases (approximately 2-fold over control) in $p42^{mapk}$ activity, consistent with a role for PP2A in the inactivation of this kinase. However, because forskolin was able to antagonize the effect of 100 nM okadaic acid on $p42^{mapk}$ activity, factors other than PP2A inhibition may be involved (e.g., preactivation of the MAPK pathway). The calcineurin antagonist FK-520 alone produced no increase in enzyme activity. Activity seen after combinations of 5 μM FK-520 with 100 nM okadaic acid was similar to that seen with 100 nM okadaic acid alone.

Fig. 8B shows the results of treatments involving NGF on $p42^{mapk}$ activity. Treatment with NGF alone increased $p42^{mapk}$ activity; this activation could be blocked by forskolin. The combination of NGF and 100 nM okadaic acid produced the greatest increase in $p42^{mapk}$ activity; treatment with forskolin was able to partially blunt the response, albeit not to basal activity levels. The NGF/FK-520 combination treatment resembled activity seen with NGF alone, in terms of both $p42^{mapk}$ activation and extent of forskolin sensitivity.

In one experiment, $p42^{mapk}$ was immunoprecipitated from 250 μg of total protein from NGF- and okadaic-treated rat brain slices. Using PhosphorImager analysis, NGF alone (100 ng/ml) increased phosphate incorporation 1.12-fold, whereas okadaic acid (10 μM) increased incorporation 1.72-fold. The combination of okadaic acid and NGF increased

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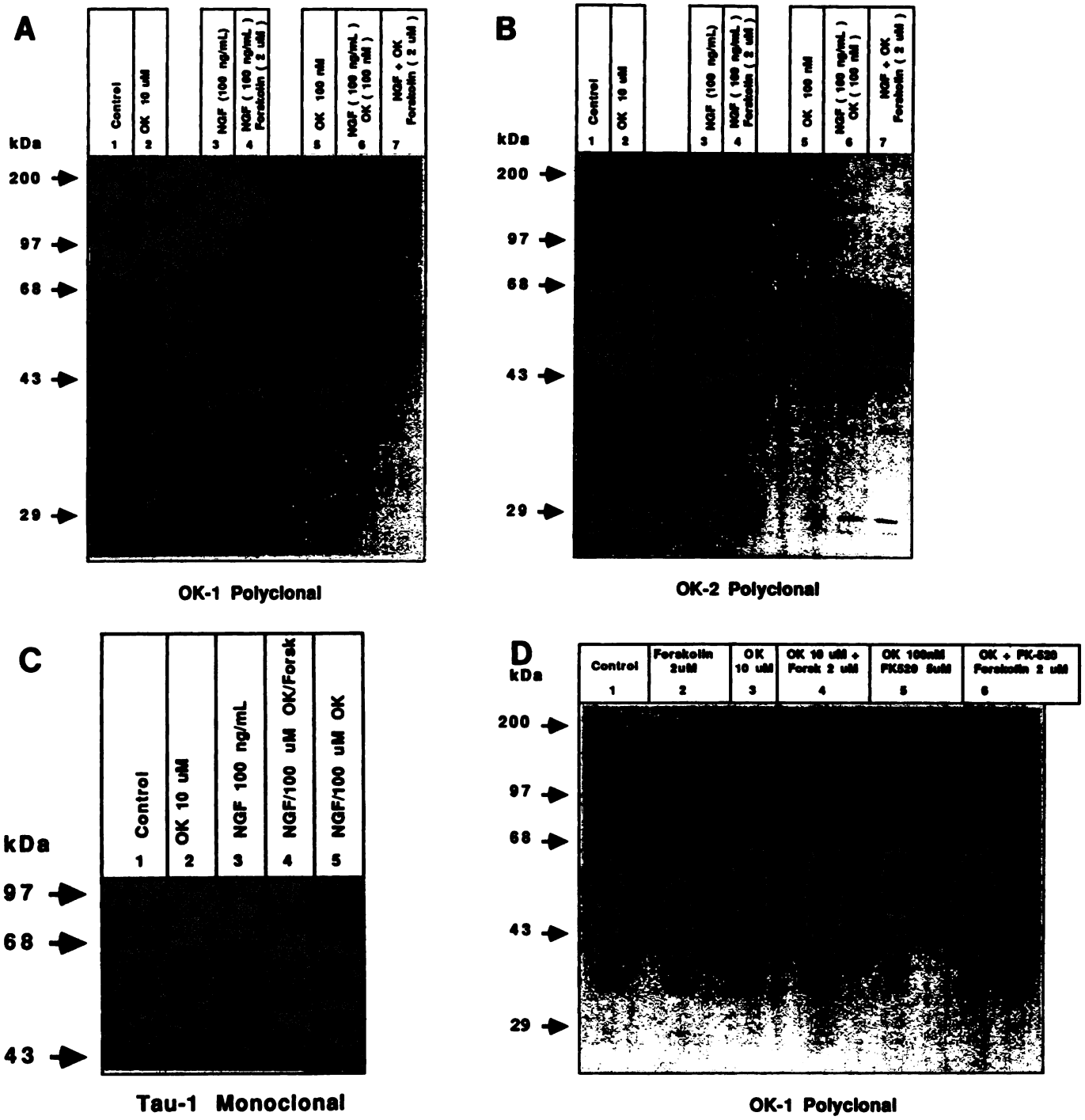

Fig. 5. Effect of forskolin on tau phosphorylation mediated by NGF, okadaic acid, and FK-520 in rat temporal neocortical slices. A and B, Rat temporal neocortical slices were prepared and treated as described in Experimental Procedures; resolved proteins were transferred to nitrocellulose membranes and incubated with OK-1 (A) or OK-2 (B) polyclonal antiserum. *Lane 1*, independent sample incubated with DMSO vehicle (control); *lane 2*, sample showing the effects of 10 μM okadaic acid (*OK*) on the development of PHF-like tau in rat temporal lobe slices; *lane 3*, sample showing the minimal effect of NGF (100 ng/ml) alone on tau mobility; *lane 4*, sample showing the effect of 2 μM forskolin plus NGF (100 ng/ml) on tau mobility; *lane 5*, sample demonstrating the lack of effect of 100 nM okadaic acid on PHF-like tau development; *lane 6*, sample demonstrating the development of PHF-like tau after a combination treatment with NGF and 100 nM okadaic acid; *lane 7*, sample demonstrating that 2 μM forskolin eliminates the ability of NGF and 100 nM okadaic acid to induce shifts in tau mobility. C, The Tau-1 monclonal antibody recognition pattern of tau isoforms after SDS-PAGE and immunoblotting is shown. *Lane 1*, sample showing the lack of effect of DMSO vehicle on tau phosphorylation (control); *lane 2*, sample demonstrating that Tau-1 does not recognize the highest molecular mass isoforms of tau after 10 μM okadaic acid treatment but does detect more subtle shifts in lower molecular mass isoforms; *lanes 3* and *4*, samples showing the lack of effect of NGF (100 ng/ml) and NGF/100 nM okadaic acid/2 mM forskolin, respectively, on tau mobility; *lane 5*, sample demonstrating the inability of Tau-1 to recognize the highest molecular mass tau isoform, although it does detect upward shifts of lower molecular mass isoforms. D, Effects of phosphatase inhibitors and forskolin in rat temporal lobe slices (detected by the OK-1 polyclonal antiserum) are shown. *Lane 1*, independent sample incubated with DMSO vehicle (control); *lane 2*, sample showing the lack of effect of 2 μM forskolin on tau mobility; *lane 3*, sample showing the effect of 10 μM okadaic acid on tau phosphorylation and mobility; *lane 4*, sample showing the lack of effect of forskolin on okadaic acid-induced shifts in tau mobility; *lane 5*, sample showing the effect of a combination of 5 μM FK-520 and 100 nM okadaic acid on tau mobility; *lane 6*, sample showing the lack of effect of forskolin on the FK-520/okadaic acid combination-induced shifts in tau mobility.

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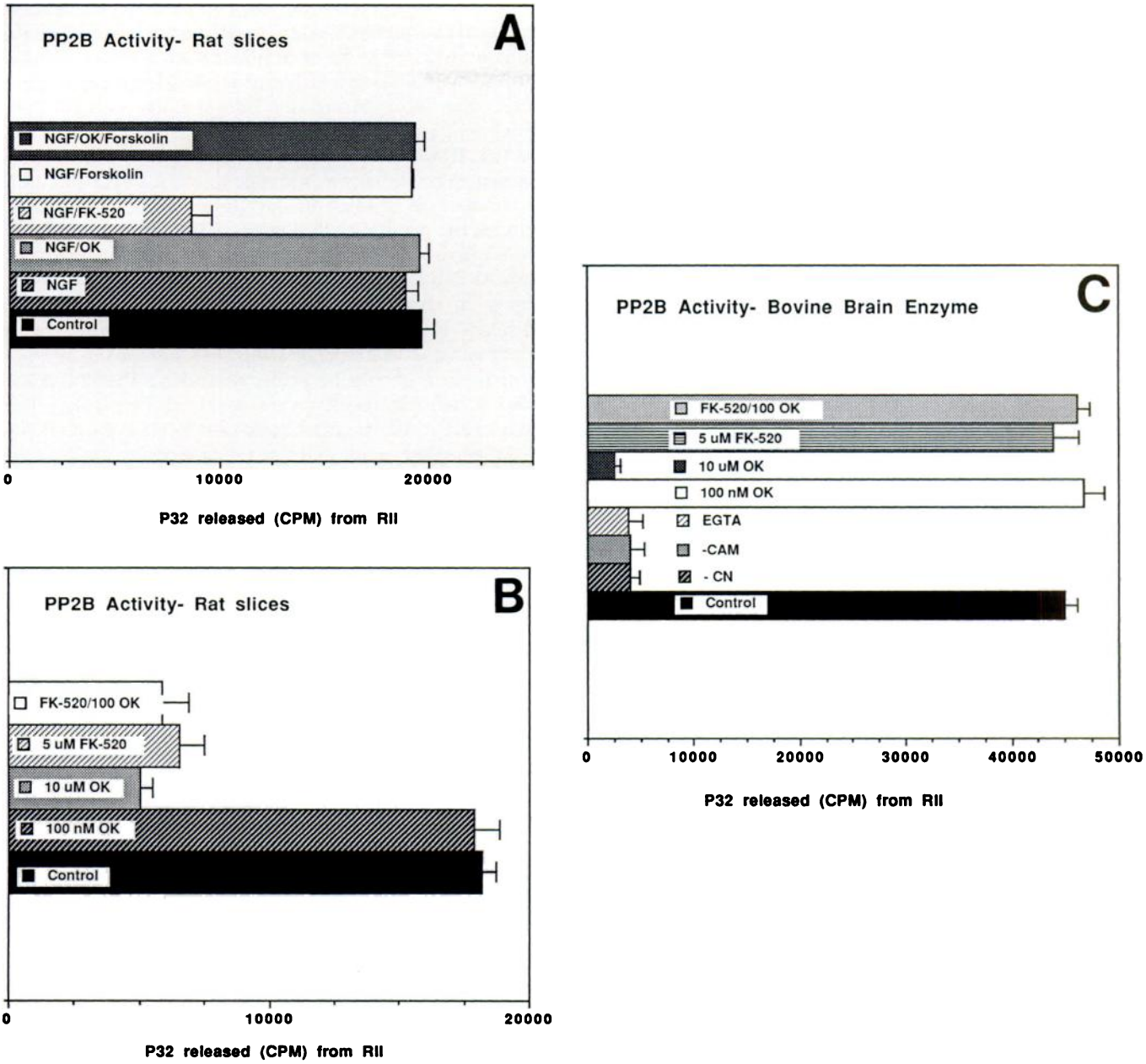

Fig. 6. Calcineurin activity in rat brain slices after inhibitor treatments. A, Calcineurin activity was measured after treatment of rat temporal lobe slices with NGF, alone and in combination with other agents. NGF alone did not inhibit calcineurin activity; however, 5 μM FK-520 did inhibit activity. B, Rat temporal lobe slices were incubated with the listed agents and homogenized, and 100 μg of total protein were added to each assay. Activity was measured as cpm of ^{32}P released (mean ± standard error) for three different rat slice experiments. Both okadaic acid (*OK*) (10 μM) and FK-520 (5 μM) inhibited calcineurin activity; okadaic acid at 100 nM had no effect. C, The effects of inhibitors on purified bovine brain calcineurin are shown. The absence of calmodulin (*CAM*) or Ca^{2+} blocked calcineurin-mediated dephosphorylation of ^{32}P-labeled R_{II} peptide, as did 10 μM okadaic acid. In the absence of exogenous FKBPs, FK-520 had no direct effect on calcineurin activity.

phosphate incorporation 1.85-fold; inclusion of forskolin reduced incorporation to 1.14 times control levels. Thus, $^{32}P_i$ incorporation into immunoprecipitable $p42^{mapk}$ roughly paralleled changes in kinase activity.

Discussion

The results presented in this study suggest that there is considerable turnover of phosphate groups on tau protein and that dual inhibition of PP2A and calcineurin can lead to accumulation of highly phosphorylated forms of tau in rat, human, and rhesus monkey brain slices. Okadaic acid consistently induced mobility shifts in tau, in a concentration-dependent manner; in a previous study, okadaic acid-induced, PHF-like tau comigrated with authentic A68 (PHF tau) from Alzheimer brain (18). Several other lines of evidence suggested that okadaic acid treatment induced formation of PHF-like tau via altered dephosphorylation. Epitope-specific monoclonal antibodies such as 5E2 and Alz-50 recognized PHF-like tau induced by phosphatase inhibitors (26, 27). Tau-1 antibody failed to detect the 68-kDa, PHF-like tau peptide. Both affinity-purified polyclonal antisera, OK-1 and OK-2, also detected changes in tau mobility

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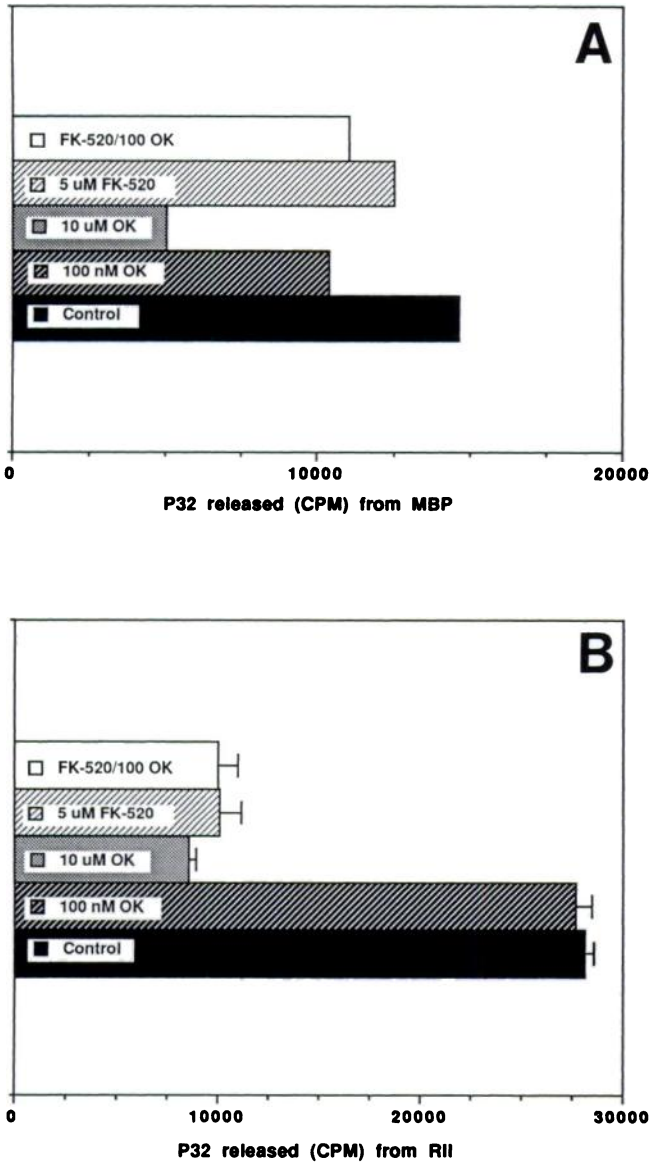

Fig. 7. PP2A and calcineurin activity in human temporal lobe slices. A, PP2A activity was measured in the same treatment groups using the release of ^{32}P from the substrate ^{32}P-labeled myelin basic protein. Treatment with both 100 nM and 10 μM okadaic acid decreased PP2A activity. B, Calcineurin activity was measured in human temporal neocortical slices after treatment with phosphatase inhibitors. Activity (cpm released; mean ± standard error) is shown for triplicate assays of the same samples. Treatment of slices with either 10 μM okadaic acid (*OK*) or 5 μM FK-520 inhibited calcineurin activity.

in rat, monkey, and human samples, including recognition of the 68-kDa peptide.

Incubation of okadaic acid-induced, PHF-like tau in human samples with alkaline phosphatase and calcineurin caused a return of higher mobility forms of tau (18),[1] resembling those of control samples; similar results were reported using alkaline phosphatase treatment of authentic A68 preparations (34). Tau is an *in vitro* substrate for calcineurin and PP2A (12, 13, 18, 19). Based on the current experiments and previous studies (7, 18, 19), we postulate that calcineurin directly dephosphorylates tau, particularly at sites phosphorylated by $p42^{mapk}$ or other proline-directed protein kinases.

If calcineurin alone were responsible for tau dephosphorylation, then inhibition of this phosphatase with FK-520, a potent analog of FK-506, would lead to accumulation of PHF-like tau. However, FK-520, at concentrations that inhibited calcineurin in slices, was not able to induce PHF-like tau in rat, human, or rhesus monkey brain slices. Explanations included the possibility that levels of FKBPs in brain may be low, relative to levels of calcineurin; this would suggest that complete inhibition of calcineurin via FK-520 is difficult to achieve in slices. However, studies on rodent brain have indicated that FKBP/calcineurin molar ratios are >1 throughout the brain, suggesting that significant inhibition of calcineurin should be achievable using FK-520 (35). In addition, similar results were obtained in monkey brain slices treated with the pyrethroid insecticide cypermethrin, a presumed selective inhibitor of calcineurin.

Two possibilities could explain the lack of effect of calcineurin inhibition on PHF-like tau formation; first, other phosphatases (e.g., PP2A) may dephosphorylate tau at sites that overlap with that of calcineurin (1) and, second, PHF tau may form only when tau-directed protein kinases are active and the protein phosphatases are inactive. To distinguish between these possibilities, rat, human, and monkey temporal neocortical slices were incubated with combinations of 5 μM FK-520 and 100 nM okadaic acid, neither of which produced tau mobility shifts when used alone. Combined treatment of temporal lobe slices with low concentrations of okadaic acid (100 nM) and FK-520 (5 μM) led to accretion of PHF-like tau in a pattern similar to that seen after addition of high concentrations (5–10 μM) of okadaic acid. Under such conditions, we found that PP2B was inhibited, PP2A was partially inhibited, and $p42^{mapk}$ was activated. We propose a dual hypothesis to explain the effect of combined treatment with okadaic acid and FK-520. First, partial PP2A inhibition by okadaic acid (100 nM) blocked dephosphorylation of Thr-183 on $p42^{mapk}$, causing activation (36). Active $p42^{mapk}$ then phosphorylated tau at Ser-Pro sites (e.g., amino acids 202, 235, and 396). Second, inhibition of calcineurin and PP2A prevented dephosphorylation of tau, leading to accretion of PHF-like tau.

It is possible that some sites on tau (e.g., Ser-46, Ser-199, Ser-202, Ser-396, and Ser-404) are dephosphorylated by both PP2A and calcineurin. Inhibition of either phosphatase alone would not be sufficient to induce PHF-like tau; however, inhibition of both PP2A and calcineurin would block dephosphorylation of tau while activating $p42^{mapk}$.

This hypothesis is further supported by experiments with NGF and okadaic acid. NGF in combination with 100 nM okadaic acid induced PHF-like tau immunoreactivity. Okadaic acid (100 nM) was able to increase $p42^{mapk}$ activity in a forskolin-sensitive manner without increasing tau phosphorylation. This indicates that activation of $p42^{mapk}$ alone is not sufficient to yield tau mobility shifts. FK-520 had no effect on $p42^{mapk}$ activity. However, NGF (100 ng/ml) and FK-520 (5 μM) produced PHF-like tau immunoreactivity. Presumably, this treatment paradigm directly activates $p42^{mapk}$ and inactivates the tau-directed phosphatase calcineurin.

Although $p42^{mapk}$ does appear to play a role in tau phosphorylation, it is clearly not the only kinase involved. Addition of forskolin to slices treated with 10 μM okadaic acid or

[1] T. D. Garver, R. A. W. Lehman, and M. L. Billingsley. Microtubule assembly competence analysis of freshly-biopsied human Tau, dephosphorylated Tau, and PHF-Tau. Submitted for publication.

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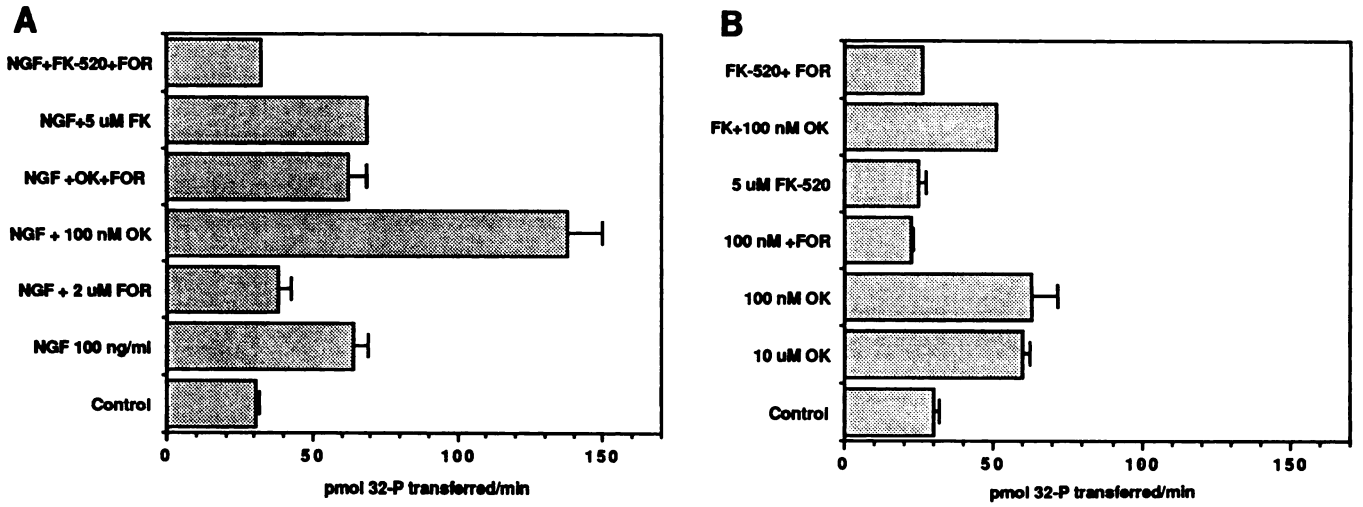

Fig. 8. $p42^{mapk}$ protein kinase activity after drug treatment in rat brain slices. $p42^{mapk}$ was immunoprecipitated and used in an activity assay as described in Experimental Procedures. Activity was measured as pmol of ^{32}P transferred to substrate/min (mean ± standard error of three separate experiments). A, $p42^{mapk}$ was immunoprecipitated and analyzed as described for B. NGF increased enzyme activity approximately 2-fold above control levels; this increase was sensitive to inhibition by forskolin. The combination treatment of NGF and 100 nM okadaic acid produced the greatest increase in activity and was only partially blunted by forskolin. The combination treatment of NGF and FK-520 resembled treatment with NGF alone in terms of both enzyme activity and forskolin sensitivity. B, Okadaic acid (*OK*) increased $p42^{mapk}$ activity at concentrations greater than 100 nM; forskolin (*FOR*) inhibited the okadaic acid-induced activity. FK-520 alone did not increase enzyme activity, but the FK-520/okadaic acid combination treatment group producedelevations in activity similar to that seen after okadiac acid treatment.

100 nM okadaic acid plus 5 μM FK-520 did not alter tau mobility. A possible explanation is that, despite decreased $p42^{mapk}$ activity, as a result of the forskolin addition other kinases (e.g., glycogen synthase kinase-β and cdk5) remain active. Consequently, these kinases continue to phosphorylate tau under conditions in which both tau-directed phosphatases (calcineurin and PP2A) are inhibited. The kinase activity is not counterbalanced and, thus, maintains a high state of tau phosphorylation.

One strength of the current study is that fresh slices of isolated temporal lobe, a region in humans that is particularly prone to development of PHFs, were maintained in a metabolically active state and used to prospectively demonstrate how phosphatase inhibition may cause accretion of PHF-like tau. Although few, if any, adequate animal models exist for the study of Alzheimer's disease, the use of human and primate brain slices affords a unique model for investigating changes in the equilibrium of tau phosphorylation. Given that PHFs form over years, one caveat is that brain slices will not allow the demonstration of PHF formation *per se*. However, it remains to be determined whether chronic treatments with selective phosphatase inhibitors can cause accumulation of PHF-like pathologies in animals.

We have recently demonstrated that tau is highly phosphorylated in normal-appearing human, rat, and primate brain *in vivo* (37, 38). Most of the presumed "PHF tau" sites, as determined with antibodies (AT8, AT270, and Alz-50), were present in rapidly biopsied human samples. These sites are rapidly dephosphorylated during postmortem intervals in non-Alzheimer brain. Thus, the activities of phosphatases are critical in maintaining a balance between PHF-like and dephosphrylated tau. A buildup of highly phosphorylated tau in Alzheimer brain may be due to a decrease in tau-directed dephosphorylation, rather than excessive protein kinase activity or *de novo* phosphorylation of aberrant sites. The current experiments suggest that PP2A and, particularly, PP2B are likely candidates as tau-directed phosphatases whose activties are needed to maintain equilibrium between functional and highly phosphorylated tau.

One possibility is that levels of phosphatases are altered in Alzheimer's disease. We recently found that levels of calcineurin immunoreactivity were unchanged in brains from patients with Alzheimer's disease, relative to age-matched controls (39). However, calcineurin was often co-localized with PHFs, raising the possibility that this enzyme is involved in cytoskeletal regulation. In addition, growth-associated protein-43, a putative substrate for calcineurin, is found in a more phosphorylated state in Alzheimer brain (40), suggesting that calcineurin activity may be lower.

It is crucial to understand how PHF-like tau forms from alterations in phosphorylation, which sites on tau are phosphorylated and dephosphorylated by specific kinases and phosphatases, respectively, and how authentic PHFs are assembled. The evidence presented in this paper supports a hypothesis whereby convergent roles of protein kinases and protein phosphatases regulate tau phosphorylation and dephosphorylation. These experiments also suggest several potential therapeutic targets for intervention to prevent PHF-like tau and neurofibrillary tangle pathology. First, agents that activate cAMP-dependent systems (e.g., forskolin) may reduce activation of the MAPK cascade. Second, selective activators of tau-directed protein phosphatases may also be of benefit in reducing neurofibrillary tangles. Finally, inhibitors of the tau-directed protein kinases also may be effective therapy in Alzheimer's disease.

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