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Activation of a neurofilament kinase, a tau kinase, and a tau phosphatase by decreased ATP levels in nerve growth factor-differentiated PC-12 cells

(Alzheimer disease/protein phosphorylation/uncouplers of oxidative phosphorylation)

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ABSTRACT **Brain pathology in Alzheimer disease and in aged controls shows hyperphosphorylation of tau and of neurofilament proteins. Roder and Ingram [Roder, H. M. & Ingram, V. M. (1991) *J. Neurosci.* 11, 3325–3343 and Roder, H. M., Eden, P. A. & Ingram, V. M. (1993) *Biochem. Biophys. Res. Commun.* 193, 639–647] previously reported that the brain protein kinase PK40^{erk} can hyperphosphorylate both tau and neurofilaments and interestingly, is strongly inhibited by ATP uncomplexed with Mg^{2+}. We now report that the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone decreases ATP levels in rat pheochromacytoma (PC-12) cells differentiated with nerve growth factor and activates a neurofilament kinase, a tau kinase, and, unexpectedly, a tau phosphatase—either PP1 or PP2A. Such aberrant modulation of protein phosphorylation patterns could be the common biochemical basis for senile dementia and for Alzheimer disease and could explain the late-onset etiology of both conditions.**

Protein phosphorylation systems are a key area of Alzheimer disease (AD) research. One of the major AD pathologies, neurofibrillary tangles, involves a defect in protein phosphorylation (1). Tangles are composed of an insoluble, hyperphosphorylated form of the microtubule-associated protein tau (2), but there is to date no indication of an inherited factor to influence this hyperphosphorylation. The kinases and phosphatases responsible for AD pathologies are the subject of much current research. The other major AD pathology, excessive formation of β-amyloid protein, may be influenced by genetic factors in certain familial forms of AD (e.g., ref. 3). In addition, hyperphosphorylation of neurofilament (NF) proteins is also a feature of old age and of AD (4, 5).

We have reported (6, 7) that a member of the mitogen-activated protein kinase family, PK40^{erk}, at relatively low concentrations of ATP can hyperphosphorylate tau *in vitro* to resemble the form found in the paired helical filaments (PHF) of AD. *In vitro* this hyperphosphorylation is strongly inhibited by elevated concentrations of ATP uncomplexed with magnesium (8). We proposed that the lower ATP concentrations in old age and in AD (9–11) allow the hyperphosphorylation of tau seen in neurofibrillary tangles. Given this expectation, we proposed (6, 7) that AD-related or age-related ATP reduction could activate PK40-like kinases and contribute to AD-like protein phosphorylation defects. Also, the mitochondrial uncoupler carbonyl cyanide *m*-chlorophenylhydrazone lowers ATP and causes an increase in AD-type antibody reactivity in human fibroblasts (12).

We now report that the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) lowers internal ATP concentrations considerably in rat pheochromacytoma (PC-12) cells differentiated to a neuronal phenotype with nerve growth factor (NGF). As a result a NF kinase, a tau kinase and a tau phosphatase become activated. We have identified the phosphatase as PP1 or PP2A, based on its sensitivity to the phosphatase inhibitors okadaic acid and calyculin A. Furthermore, in the presence of okadaic acid, FCCP increases the activity of an endogenous tau kinase, leading to hyperphosphorylated tau resembling AD-tau of PHFs. Our results suggest that ATP levels can play a broad role in modulating the activity of both protein phosphatases and kinases.

EXPERIMENTAL PROCEDURES

ATP Assays. PC-12 cells (American Type Culture Collection) were plated on collagen-coated plates at 4×10^4 cells per cm^2 and maintained in a humidified, 37°C, 5% CO_2/95% air incubator. Cells were grown in RPMI 1640 medium/2 mM L-glutamine/penicillin at 50 units/ml/streptomycin at 50 μg/ml/10% donor horse serum (JRH Biosciences, Lenexa, KS)/5% bovine calf serum (HyClone) for 3 days and then switched to a low-serum medium (as above except with 1% donor horse serum only) with NGF at 50 ng/ml (Promega 2.5S murine) for 6 days. When used, FCCP was diluted into fresh low-serum, NGF-supplemented medium to a final concentration of 30 μM. All controls received equivalent vehicle, 0.1% ethanol. At each time point, cultures were lysed in 10 ml of 0.4 M $HClO_4$, incubated on ice 1 hr, and centrifuged. Two hundred microliters of supernatant was mixed with 100 μl of neutralizing solution (0.72 M KOH/0.6 M $KHCO_3$), kept at −20°C for 10–14 hr, thawed, and centrifuged. The ATP-containing supernatant was assayed by using a luciferin–luciferase ATP determination kit (Sigma). Light emitted was measured in a Beckman LS7500 liquid scintillation counter, out of coincidence, and with a fully open window.

Tau Dephosphorylation and NF Phosphorylation. Cell culture and FCCP treatment were done as above, except that PC-12 cells were plated at 2×10^4 cells per cm^2, grown in high-serum medium/NGF for 6 days and then in low-serum medium/NGF for 4 days. Control cells received equivalent vehicle, 0.1% ethanol. Cells were lysed in 400 ml of buffer (25 mM Na_2HPO_4/0.4 M NaCl/0.5% SDS/10 mM benzamidine·HCl/1 mM phenylmethylsulfonyl fluoride *o*-phenanthroline at 1 mg/ml/ aprotinin at 10 mg/ml, leupeptin at 10 mg/ml,

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Abbreviations: AD, Alzheimer disease; NF, neurofilament; NGF, nerve growth factor; PHF, paired helical filament(s); FCCP carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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pepstatin A at 10 mg/ml, sonicated, boiled for 5 min, and centrifuged. Aliquots of the supernatant (30 μg of protein per lane) were loaded on SDS gels, transferred to Immobilon-P, blocked overnight in 5% nonfat dry milk, and immunoblotted with primary monoclonal antibody (mAb) at 1:1000 [Tau-1, Boehringer Mannheim; SMI-34, Sternberger–Meyer, (Jarrettsville, MD); RM0-44, Zymed] or 1:4000 (anti-tau polyclonal antibody, Sigma). Secondary antibodies, a goat anti-mouse IgG/A/M peroxidase conjugate or a goat anti-rabbit IgG peroxidase conjugate (Sigma), were used at 1:3000 and 1:4000, respectively. Blots were developed by using the Amersham enhanced chemiluminescence kit.

Effect of FCCP and of Okadaic Acid on Tau Dephosphorylation (See Fig. 3). PC-12 cells were preincubated with 1.0 μM okadaic acid for 15 min. Medium was then replaced by FCCP, okadaic acid, or FCCP/okadaic acid supplemented medium for 2 hr. In all cases, control cells were treated with equivalent vehicle/0.15% ethanol. For alkaline phosphatase treatment, blots were incubated in 3 ml of 0.1 M Tris·HCl, pH 8.0 with calf intestine alkaline phosphatase type VII-S at 100 units/ml (Sigma) at 37°C for 17 hr. SDS/PAGE and immunoblotting were done, as described earlier.

Effect of FCCP and of Okadaic Acid on Tau Dephosphorylation and NF Phosphorylation (See Fig. 4). PC-12 cells were preincubated with 0.5 μM okadaic acid for 15 min. FCCP was then added directly to each culture dish to a final concentration of 30 μM. Protein extracts were made at the specified times. The control received equivalent vehicle, 0.15% ethanol, for 1 hr. Ethanol did not provoke protein phosphorylation changes at any of the time points used (data not shown). Thirty micrograms of total protein was loaded per lane on SDS/10% acrylamide gels and immunoblotted.

RESULTS

ATP Depletion by FCCP. To investigate the effects of ATP depletion we used the mitochondrial uncoupler FCCP (13) to lower ATP levels in NGF-differentiated PC-12 cells in low serum medium. ATP levels fell to ≈65% of control values after 0.5-hr treatment with 30 μM FCCP and remained depleted over the next 4 hr (Fig. 1). As indicated by the gradual lowering of pH in the medium, indicative of lactic acid production, the cells seem to switch to glycolysis to make their ATP, although at a lower rate.

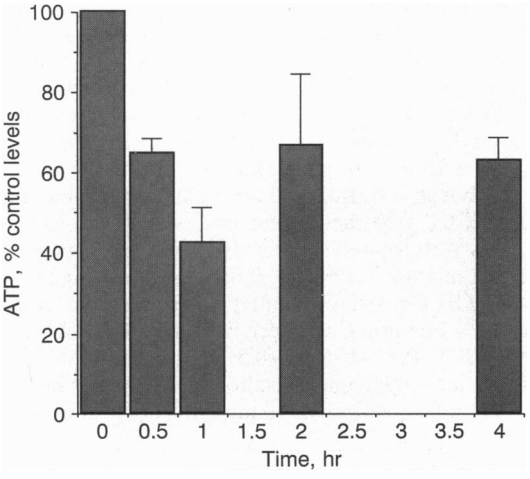

FIG. 1. ATP is rapidly depleted in PC-12 cells by 30 μM FCCP. ATP levels fell to 65% of control values after 0.5 hr of FCCP treatment (earliest time point examined) and remained depleted during the next 4 hr. Values are expressed as percentage of control ATP levels and are the average of three separate determinations. Controls were measured in duplicate for each time point. Error bars indicate 1 SD.

Activation of a NF Kinase by FCCP. We assayed the phosphorylation state of tau and NFs during FCCP treatment with the phosphorylation-sensitive mAbs Tau-1 and SMI-34. mAb Tau-1 binds only to nonphosphorylated Ser-Pro motifs in residues 192–204 of htau40, the longest recombinant human tau isoform (14). Normal human tau is reactive with mAb Tau-1 but loses reactivity as tau becomes phosphorylated in AD (15). mAb SMI-34, in contrast, is a marker for hyperphosphorylation. Normal human brain sections do not stain with mAb SMI-34, but both AD neurofibrillary tangles and normal aged brains are mAb SMI-34 reactive (16). mAb SMI-34 has been shown to bind to the medium and heavy NF subunits (NF-M and NF-H) at their multiple Lys-Ser-Pro phosphorylation repeats (17), as well as to hyperphosphorylated tau at residues surrounding the microtubule-binding domain (1).

We exposed differentiated PC-12 cultures to 30 μM FCCP in a low serum medium for 30 min–7 hr and monitored the phosphorylation state of tau and NF proteins. As predicted, phosphorylation of NF-M and NF-H increased markedly as measured by increased immunostaining with mAb SMI-34 (Fig. 2*c*). To confirm that the increased mAb SMI-34 staining was due to NF phosphorylation and not to changes in total protein levels, we blotted portions of the same extracts with mAb RMO-44, a phosphate-independent antibody to NF-M (19); mAb RMO-44 stained all samples with equal intensity (Fig. 2*d*).

In other experiments (see Fig. 4*b*), the phosphatase inhibitor okadaic acid greatly increased the FCCP-induced NF phosphorylation. Both FCCP and, to a lesser extent, okadaic acid, cause increased NF phosphorylation. When FCCP and okadaic acid are applied together, phosphorylation increased greatly.

To address whether the uncoupler FCCP acts via ATP depletion or via other, unknown pathways, we tested the effects of the ATP synthase inhibitor oligomycin (data not shown). Oligomycin, like FCCP, is known to deplete cellular ATP levels but is not associated with dissipation of mitochondrial electrochemical gradients. At 50 μM, oligomycin had similar effects on NFs as did FCCP. By 1 hr of oligomycin treatment there was an increase of NF phosphorylation, as measured by mAb SMI-34 staining (Tau dephosphorylation also occurred; see below). The similarity of the FCCP and oligomycin results supports ATP depletion as a possible mechanism.

Activation of a Tau Phosphatase by FCCP. When we examined the effects of 30 μM FCCP in low serum medium on the phosphorylation status of tau, we saw tau dephosphorylation, indicated by a large increase in mAb Tau-1 staining (Fig. 2*a*). A phosphate-independent polyclonal antibody to tau confirmed that total tau levels remain approximately constant (Fig. 2*b*). Furthermore, several tau bands show an increased gel mobility with the polyclonal antibody, consistent with dephosphorylation. FCCP at 5–10 μM also caused slight tau dephosphorylation, but 30 μM was required for the full effect (Fig. 3*a*). We interpret these results as showing that 30 μM FCCP simultaneously activates a NF kinase and a tau phosphatase. The tau kinase(s) endogenously present cannot, in the presence of FCCP activation of a phosphatase, hyperphosphorylate tau to PHF-like tau. Interestingly, using a medium containing 15% serum decreases but does not eliminate the phosphorylation/dephosphorylation effects (data not shown). The role of serum is unknown. It has been reported (12) that the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone causes an increase in AD-type antibody reactivity in human fibroblasts but only when serum was removed from the culture medium.

To identify the FCCP-induced phosphatase, we tested the effects of the serine phosphatase inhibitors okadaic acid and calyculin-A. The inhibitors are equally potent against PP2A (IC_{50} 2 nM), but calyculin-A is much more effective against

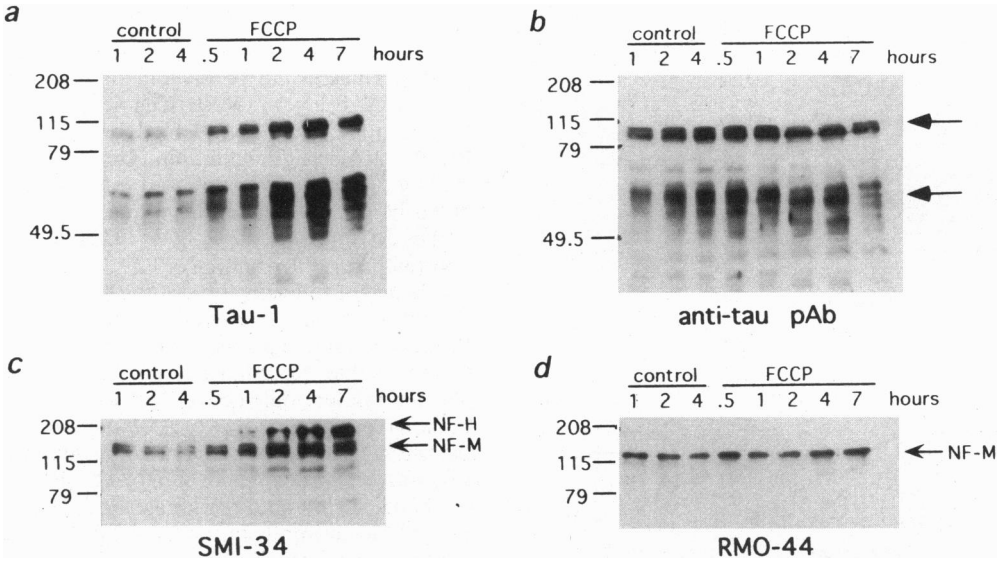

FIG. 2. FCCP induces a tau phosphatase and a NF kinase. (*a*) After 2–4 hr of 30 μM FCCP treatment, PC-12 cell extracts show increased reactivity with mAb Tau-1. The 50- to 60-kDa tau isoforms are visible, as well as a high-M_r tau doublet found only in the peripheral nervous system (18). (*b*) A phosphorylation-independent anti-tau polyclonal antibody confirms that total tau levels remain approximately constant while mAb Tau-1 staining increases dramatically. Several bands run at altered positions (arrows), consistent with a dephosphorylation-induced increase in gel mobility. (*c*) Staining with mAb SMI-34 shows greatly increased phosphorylation of NF proteins NF-M and NF-H. (*d*) Immunoblotting with the phosphate-independent mAb RMO-44 confirms that total NF-M levels do not change. The basic observations of *a* and *c* have been repeated at least four times, and the other experiments in Figs. 2–4 are in part replicates.

PP1 (IC_{50} 2 nM for calyculin A and 100 nM for OA) (20). Neither inhibitor is effective against PP2B (calcineurin) at concentrations <5 μM (21). FCCP-induced tau dephosphorylation was completely blocked by 0.5–1.0 μM okadaic acid (Figs. 3*b* and 4*a*) and 0.1 μM calyculin A (data not shown). This result suggests that FCCP activates PP1 and/or PP2A, possibly

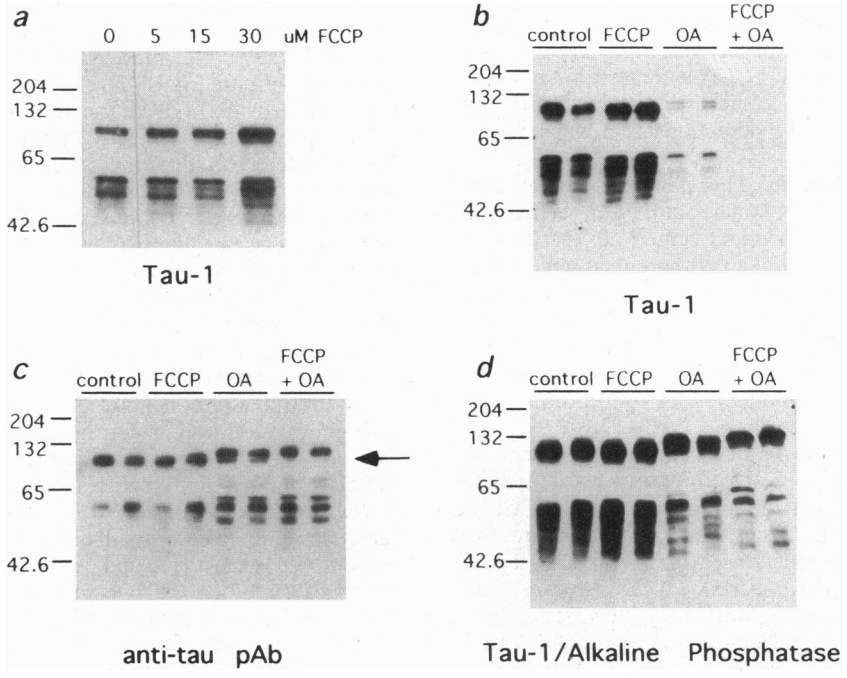

FIG. 3. Increased tau phosphatase activity is maximal at 30 μM FCCP and is blocked by the PP1/PP2A inhibitor okadaic acid. In the presence of okadaic acid, FCCP activates a tau kinase. (*a*) mAb Tau-1 immunoblot showing increasing tau dephosphorylation with increased FCCP concentrations. All treatments were for 2 hr; 1 μM and 0.1 μM FCCP had no effect (data not shown). (*b–d*) Cells were treated for 2 hr with 30 μM FCCP, 1 μM okadaic acid (OA), or both. Duplicate extracts are shown for each condition. (*b*) mAb Tau-1 immunoblot showing FCCP-induced phosphatase activation is blocked by okadaic acid. Okadaic acid alone causes tau phosphorylation (decreased mAb Tau-1 staining), but phosphorylation is greater when FCCP is also present. (*c*) The phosphate-independent tau polyclonal antibody confirms that changes in mAb Tau-1 staining are not due to changes in total tau levels. Tau phosphorylation is reflected by decreased gel mobility in the okadaic acid and FCCP plus okadaic acid samples. FCCP and okadaic acid together cause a greater gel shift of tau than okadaic acid alone, shown in the complete shift of the lower band of the high-M_r tau doublet (arrow). (*d*) Treatment of the blot with alkaline phosphatase before mAb Tau-1 staining recovers most of the mAb Tau-1 epitope in the okadaic acid and FCCP plus okadaic acid samples but does not affect other phosphorylated sites. Again, gel shift of phosphorylated tau is greater in the FCCP plus okadaic acid samples than with okadaic acid alone.

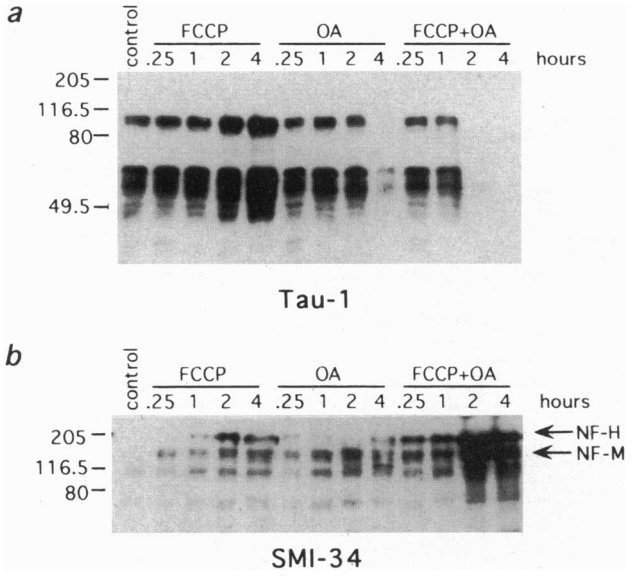

FIG. 4. Time course of the increase in tau kinase and NF kinase activities by FCCP in the presence of okadaic acid (OA). (*a*) Time course of FCCP- and okadaic acid-induced changes in tau phosphorylation in 30 μM FCCP, 0.5 μM OA, or both; extracts were blotted with mAb Tau-1. (*b*) Time course of FCCP- and okadaic acid-induced NF phosphorylation. Aliquots of the same extracts as in *a* were blotted with mAb SMI-34.

only PP1, because calyculin-A blocked phosphatase activation more effectively than okadaic acid. No effect on tau phosphatase activation was seen with 0.1 μM deltamethrin, specific inhibitor for calcineurin (PP2B) (IC_{50} 1 nM–10 pM).

At 50 μM, oligomycin had similar effects on tau dephosphorylation as did FCCP but with a longer time course. mAb Tau-1 staining relative to control increased only after 7 hr of oligomycin treatment, whereas FCCP increased mAb Tau-1 staining by 2 hr. Again, the similarity of the FCCP and oligomycin results supports ATP depletion as a possible mechanism.

Activation of a Tau Kinase. The inhibitor studies also showed that in the presence of okadaic acid, FCCP activates a tau kinase (Fig. 3 *b–d*). Okadaic acid alone (1.0 μM) caused a loss of mAb Tau-1 reactivity and an upward gel mobility shift characteristic of hyperphosphorylated tau; both effects were increased when FCCP was also present (Fig. 3 *b* and *c*). We confirmed that the FCCP-induced loss of the mAb Tau-1 epitope was due to tau phosphorylation by showing that alkaline phosphatase treatment of the blot restored the mAb Tau-1 epitope (Fig. 3*d*).

After 4-hr treatment with okadaic acid, tau has become hyperphosphorylated due to reduced phosphatase activity (Fig. 4*a*); it hardly reacts with mAb Tau-1. When FCCP is added in addition to okadaic acid, a kinase phosphorylates tau more quickly and more completely; after 2 hr, mAb Tau-1 reactivity has completely disappeared. FCCP also increased the speed with which tau phosphorylation occurs; 0.5 μM okadaic acid alone caused a decrease in the mAb Tau-1 epitope after 4-hr treatment, but in the presence of FCCP, mAb Tau-1 reactivity was completely abolished by 2 hr (Fig. 4*a*). Clearly, the presence of the phosphatase inhibitor allows endogenously active kinases to hyperphosphorylate tau at the Tau-1 site. The gel shift pattern seen with the general anti-tau polyclonal antibody in the presence of okadaic acid resembles the pattern characteristic of human tau as extracted from AD tangles/PHF (Fig. 3*c*). The gel shift seen with the large tau band is also characteristic of hyperphosphorylation. Only some of those phosphorylated sites can be removed by alkaline phosphatase (Fig. 3*d*).

DISCUSSION

Our results indicate that FCCP can modulate the activity of protein kinases and of protein phosphatases. When FCCP reduces ATP levels, the striking activation of the NF kinase, for example, can be explained by the expected release of $PK40^{erk}$ from ATP inhibition. However, other explanations are conceivable. The increased NF kinase activity observed might be due to increased synthesis of the kinase.

We do not yet know how FCCP activates the protein phosphatases PP1 and/or PP2A, or why okadaic acid is necessary for full activation of the tau kinase. It is known that members of the mitogen-activated protein kinase family (ERKs) require phosphorylation on both threonine and tyrosine residues for full activation (22). Only the appropriately phosphorylated, fully active form of the kinase is expected to be capable of responding to ATP depletion. It is possible that the tau kinase may be inactivated by dephosphorylation when PP1 and/or PP2A are activated by FCCP. Only in the presence of okadaic acid could the kinase be fully phosphorylated and susceptible to upregulation by ATP depletion. Activation of phosphatases might be understood in the light of reports that PP2A is inhibited by ATP (23); the inhibition is abolished by addition of Mn^{2+}. Alternatively, an activated kinase, like $PK40^{erk}$ might phosphorylate and thereby activate the phosphatase.

Although we think that FCCP acts through ATP depletion, FCCP is also known to raise intracellular calcium (24) and may have other, unknown effects on the cell. A preliminary experiment with the calcium ionophore A23187 in 0.4 mM external calcium indicates that calcium influx does not mimic the effects of FCCP in our system.

Both the tau and NF kinases are FCCP sensitive, phosphorylate Ser-Pro sites, and can act at the mAb Tau-1 and mAb SMI-34 epitopes, respectively. Enzymes known to generate these epitopes are the Alzheimer "candidate kinases," which include $PK40^{erk}$, p42 mitogen-activated protein kinase (25), glycogen-synthase kinase 3 (26, 27), cdk2 and cdk5 (28), and a tubulin-activated tau kinase (29). Of these only $PK40^{erk}$ is known to become more active when ATP is reduced. To the best of our knowledge, the other kinases have not yet been tested.

The activation of NF kinases, as seen in Fig. 4*b*, is interesting because it suggests that two distinct NF kinases become active in FCCP and in okadaic acid. Both conditions produce a large increase in NF phosphorylation as detected by mAb SMI-34. However, the patterns differ. In FCCP after 2 hr the large NF-H protein is more heavily phosphorylated than the intermediate NF-M protein. In okadaic acid after 1 or 2 hr NF-M is by far the more heavily phosphorylated; in fact, NF-H phosphorylation only becomes apparent after 4 hr (Fig. 4*b*). The NF kinase activated in the presence of the phosphatase inhibitor okadaic acid shows a substrate specificity pattern that is characteristic of $PK40^{erk}$ (1), whereas the FCCP-induced pattern is quite different and unknown. When both agents are present, the pattern seen appears to be the sum of both kinases. Also, the PHF-like tau phosphorylation pattern seen by the polyclonal anti-tau antibody (Fig. 3*c*) in cell cultures treated with okadaic acid alone is characteristic of $PK40^{erk}$ (2).

Although AD is a complex disorder, the observation that kinase and phosphatase activities can be altered by decreased ATP levels suggests an explanation for the late-onset etiology of this disease and of senile dementia. Some observers have reported that oxidative metabolism and therefore ATP production become increasingly impaired with age (11) and more markedly impaired in AD (9, 10). The known age-dependent accumulation of mitochondrial DNA mutations (6, 30, 31) would inactivate several essential mitochondrial enzymes. Nondividing cells, such as neurons, would accumulate a certain proportion of dysfunctional mitochondria. This accumulation

might be a contributing factor to the etiology of AD and of "normal" brain aging because it would be expected to lower ATP levels. As a consequence, the phosphorylation status of cytoskeletal proteins would be greatly altered, as seen by us in PC-12 cells, probably with deleterious effects on the neurons of the aging and of the AD brain.

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