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Decreased levels of protein kinase C in Alzheimer brain

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Protein kinase C (PK-C) levels were determined using [^{3}H]phorbol-12,13-dibutyrate (PDB) binding and the in vitro phosphorylation of histone H I (III-S), in autopsied human frontal cortex of age- and postmortem time-matched normal and Alzheimer patients. PK-C levels in Alzheimer particulate fractions determined by both methods were about 50% of those in controls. PK-C levels in Alzheimer cytosol fractions were not significantly different from those in controls. In a parallel study, we measured the phosphorylation of a M_r 86,000 protein (P86), the major protein kinase C substrate in the cytosol fraction prepared from Alzheimer frontal cortex, and found it to be reduced to 43% of that in control brains. This reduction in P86 protein phosphorylation compared to controls was not detected in brain samples prepared from demented patients without Alzheimer's disease. We considered 3 extraneous factors (postmortem delay, age and sex) which may have affected the extent of P86 phosphorylation and concluded that the reduced P86 phosphorylation in the Alzheimer samples is not due to any of them. Reduced PK-C levels and M_r 86,000 protein phosphorylation may reflect a biochemical deficit related specifically to the pathogenesis of Alzheimer's disease.

INTRODUCTON

One of the characteristics of Alzheimer's disease is the selective loss of large neurons in the cerebral cortex[59]. Several neurotransmitters, their marker enzymes, and certain neurotransmitter receptors are reduced in brain tissue from Alzheimer patients. These include acetylcholine[9,15,35,45,57], somatostatin[7,14,50], norepinephrine[12], serotonin[11], substance P[13], glutamate[21], corticotropin releasing factor[8,16], and neuropeptide Y[6]. Some of these neurotransmitters have been co-localized with neuritic plaques and neurofibrillary tangle-bearing neurons, well-documented morphological abnormalities found in Alzheimer brains[4,5,40,49]. It may be, therefore, that the large neurons containing these neurotransmitters and receptors are the ones dying in the Alzheimer neocortex. Moreover, this loss of neurons is relatively selective. Concentrations of many other neurotransmitters and their receptors have been found to be unchanged. Among them are dopamine, γ-aminobutyric acid[44], cholecystokinin[52], vasoactive intestinal peptide[51], and neurotensin[17]. Further, neuronal populations in the basal ganglia and cerebellum are relatively intact in Alzheimer patients.

Selective deficits in neurotransmitter metabolism are not likely to cause cell death. Why then do the cortical neurons die? No biochemical deficits have yet been reported to explain this neuronal death. However, there are interesting hypotheses. In 1981, Appel proposed the lack of certain neurotrophic factors may be responsible for neuronal death in the brain of Alzheimer patients[3]. Two years later, Hefti proposed that the lack of nerve growth factor (NGF) may be responsible for the cholinergic neurodegeneration in Alzheimer patients[23]. However, normal levels of NGF mRNA were found in Alzheimer patients[20], and the neuronal degeneration is not restricted to cholinergic cells supported by NGF. Alternatively, it is possible that neurons which die in the

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Alzheimer brain may be well supplied with necessary growth factors but fail to respond to these factors because of a deficiency in receptors or intracellular signaling machinery. Because several classes of neurons with apparently different neurotrophic factors are involved, we have focused on postreceptor mechanisms, common to several trophic factors.

Phosphorylation reactions have long been known to be important in mediating the function of growth factors[10,27,41,42], and it has been suggested by several groups that phosphorylation reactions in Alzheimer neurons are aberrant[22,53,58]. This suggested the possibility that an abnormal phosphorylation reaction in Alzheimer neurons is responsible for neuronal death, because cells which cannot respond properly to extracellular signals such as growth factors required for survival, will die.

Among the many kinases, the Ca^{2+}/phospholipid-dependent protein kinase (also known as protein kinase C or PK-C) plays an important role in the control of cell vitality, growth, and differentiation[43]. Phorbol ester, an activator of PK-C, has been reported to mimic several neurotrophic factors, including NGF, in supporting survival of some neurons[24,25,39], suggesting the special importance of this enzyme in the nervous system. In fact, this kinase is more abundant in brain tissue than elsewhere[31,37], and can be detected in postmortem human brain cytosol[54]. Because of its critical role in neuronal survival, we chose to study PK-C in brain tissues of Alzheimer patients and in non-Alzheimer controls. We report here a reduction in Alzheimer brain in PK-C levels and in the in vitro phosphorylation of a M_r 86,000 protein (P86), a major protein kinase C substrate.

MATERIALS AND METHODS

Brain tissue pathology

Brains were removed after death and divided sagittally in the midline. The right hemibrain was frozen at −70 °C for biochemical analysis and the left hemibrain was placed in 10% formalin for 1–2 weeks. The fixed hemibrains were sectioned serially in the coronal plane at 1 cm thickness and the slices examined grossly.Tissue for microscopic examination was obtained from midfrontal cortex, superior temporal gyrus, inferior parietal cortex, inferior temporal gyrus, precentral gyrus, cingulate gyrus, striate cortex, substantia innominata, hippocampus, amygdala, mesencephalon, pons, and cerebellum. Hematoxylin and eosin preparations from each tissue block were made for overall histopathologic evaluation. Thioflavine-S stains, examined by fluorescence microscopy, were employed to visualize and quantify neuritic plaques, neurofibrillary tangles, and cerebrovascular amyloid.

The cases in this study were divided into three diagnostic categories. In 26 cases, the patients were clinically demented and neuropathologic evaluation confirmed the clinical diagnosis of Alzheimer's disease. Eight demented patients were found to have various other causes for dementia, including Pick's disease (3 cases), Parkinson's disease without accompanying Alzheimer's disease (2 cases), Binswanger's disease (1 case), multi-infarct dementia (1 case), and hippocampal sclerosis (1 case). A third group of 16 cases included patients without either clinical dementia or significant neuropathologic abnormalities. Criteria for the diagnosis of Alzheimer's disease included the presence of numerous neuritic plaques and neurofibrillary tangles in the midfrontal cortex (corresponding to the tissue analyzed for PK-C from the contralateral hemisphere). The 16 cases without Alzheimer's disease, clinical dementia, or significant neuropathology were used as controls in the following study.

Biochemical procedures

Procedures for the preparation of the cytosol fraction and the conditions for phosphorylation in the presence of PK-C activators (phorbol 12-myristate 13-acetate (PMA), and phosphatidylserine (PS)) have been described previously[54]. Human midfrontal cortex was dissected from 1-cm-thick sections of frozen tissue on a glass plate cooled from below by a bed of powdered dry ice. Dissected cortex was placed in 10 vol of homogenization buffer (0.32 M sucrose/5 mM HEPES, pH 8.0/5 mM benzamidine/2 mM 2-mercaptoethanol/3 mM EGTA/0.5 mM $MgSO_4$/5 mM glycerophosphate/5 mM potassium fluoride/10 μM sodium vanadate/0.1 mM phenylmethylsulfonyl fluoride/leupeptin (0.1 mg/ml)/pepstatin (0.05 mg/ml)/aprotinin (0.1 mg/ml)), homogenized by two 5-s strokes of a Polytron homogenizer (Brinkman), and centrifuged 1 h at 100,000 *g* at 2 °C to separate particulate fractions (100,000 *g* pellet) from cytosol.

Ten μl of each fraction in the homogenization buffer containing 6.5 μg total protein was mixed with 15 μl of reaction mixture containing 50 mM Tris-HCl, pH 7.6 (all concentrations are for final 25-μl volume), 10 mM $MgSO_4$, 100 μg protein kinase A inhibitor/ml (Sigma), 5 mM 2-mercaptoethanol, 10 μM ATP, [γ-^{32}P]ATP (1 μCi per tube; 1 Ci = 37 GBq) (ICN), and 0.2 mM EDTA. Histone III S (Sigma), 20 μg/tube, was added for the determination of PK-C activity. To assess the PK-C dependent phosphorylation, two series of assays were with PK-C activators (0.1 μM PMA and 50 μg/ml PS) and two series were without them. The mixture underwent reaction for 1 min to determine PK-C activity with histone and for 12 min for endogenous reaction at 30 °C. The reaction was stopped by addition of 6.3 μl of a buffer containing 40% (vol/vol) glycerol, 25% 2-mercaptoethanol, 12% SDS, 0.31 M Tris-HCl (pH 6.8), 25 mM EDTA, and 0.1% bromophenol blue. The samples which had been labeled with ^{32}P by incubation with [^{32}P]ATP were then subjected to 6.5–12.5% polyacrylamide gel (1 mm thickness) electrophoresis with 2-cm stacking gel[32] at a current of 20 mA per gel. The electrophoresis was stopped when the tracking dye reached 1 cm from the edge of the gel. Gels were fixed 1 h in 10% acetic acid containing 15% isopropyl alcohol, stained 1 h with 0.2% Coomassie brilliant blue in 10% acetic acid and 40% isopropyl alcohol, and destained in 10% acetic acid and 15% isopropyl alcohol. Destained gels were rinsed 1 h with water, dried, and exposed about 10 h on X-Omat RP film (Kodak) with Hi Plus intensifying screen (Du Pont) at −70 °C. Protein concentrations were determined using the method of Lowry et al.[33], with plasma globulin (Bio-Rad, Richmond, CA) as the standard. Calcium was not used in the current phosphorylation assay because of our initial concern that high levels of calcium may activate proteolytic activities (e.g. calpains). In our previous investigation in which we established PK-C assay conditions for postmortem human brain homogenates[54] we found that our present assay conditions (without calcium, but in the presence of phorbol ester which increases the affinity of PK-C for calcium) there was sufficient residual calcium to activate PK-C and give consistent results.

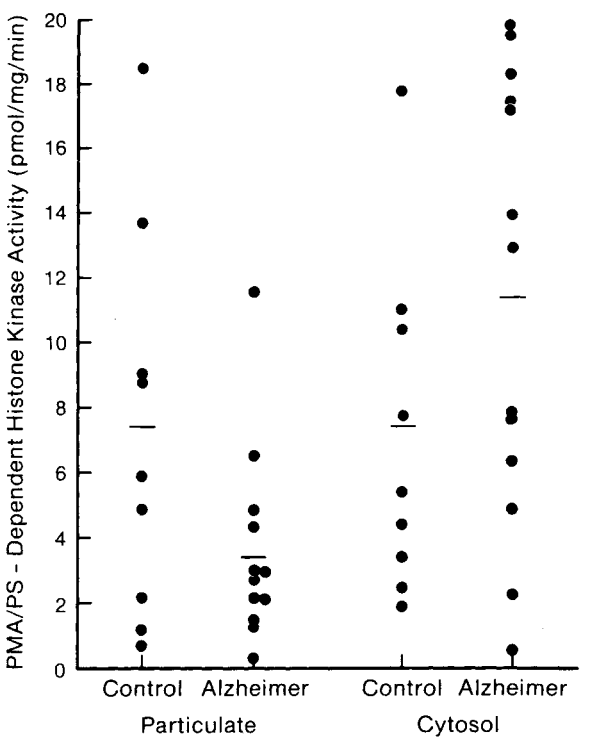

Fig. 1. Scattergram of the distribution of PMA/PS-dependent histone kinase activity. Individual data points described in Table I are plotted in two fractions from control and Alzheimer.

TABLE I

Distribution of PMA/PS-dependent histone kinase activity

Protein (6.5 μg) from each fraction was incubated with 20 μg histone I and [^{32}P]ATP in the presence or absence of PMA/PS. PMA/PS-dependent incorporation of radioactivity into histone was quantified by counting the histone band dissected from the dried gels. Values are means ± S.D. Controls were from 9 individuals diagnosed to be clinically and pathologically normal. Their mean (± S.D.) age of death was 75.4 ± 3.8 years, and their mean postmortem time was 7.8 ± 7.6 h. Alzheimer samples contain 13 cases. Their mean age of death was 74.4 ± 6.3 years, and their mean postmortem time was 7.0 ± 5.2 h.

	Particulate (pmol/mg/min)	*Cytosol (pmol/mg/min)*
Control	7.22 ± 4.05	7.32 ± 4.98
Alzheimer	3.37 ± 1.58*	11.31 ± 8.33

* Different from control, $P < 0.01$ by unpaired Student's t-test.

The amount of PK-C was measured by a modification of the phorbol ester binding method[60]. The reaction mixture contained 25 mM Tris-HCl, pH 7.5, 10 mM Mg acetate, 1.4 mM $CaCl_2$, 0.4 mM EGTA, 50 mM KCl, 4 mg/ml of bovine serum albumin, 100 μg/ml of phosphatidylserine, 2 nM [^{3}H]phorbol-12,13-dibutyrate ([^{3}H]PDB) ± 2 μM PMA in a total volume of 200 μl. PS solution was sonicated just be-

TABLE II

Distribution of [^{3}H]PDB binding activity

Protein (25 μg) from each fraction was incubated with [^{3}H]PDB in the presence of Ca^{2+} and phosphatidyl serine. The bound [^{3}H]PDB was collected on glass fiber filters. Values are means ± S.D. Controls were from 9 individuals diagnosed to be normal. Their mean (± S.D.) age of death was 75.4 ± 3.8 years, and their mean (± S.D.) postmortem time was 7.8 ± 7.6 h. Alzheimer samples contain 13 cases. Their mean (± S.D.) age of death was 74.4 ± 6.3 years, and their mean (± S.D.) postmortem time was 7.0 ± 5.2 h.

	Particulate (pmol/mg)	*Cytosol (pmol/mg)*
Control	1.59 ± 0.96	0.38 ± 0.29
Alzheimer	0.72 ± 0.24*	0.46 ± 0.30

* Different from control, $P < 0.01$ by unpaired Student's *t*-test.

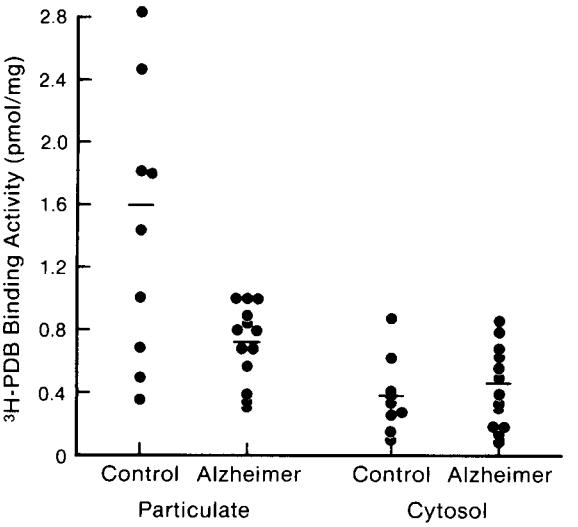

Fig. 2. Scattergram of the distribution of [^{3}H]PDB binding activity. Individual data points described in Table II are plotted in two fractions from control and Alzheimer.

fore use. After adding 40 μl of sample containing 25 μg proteins, the tubes were incubated 2 h at 4 °C. Bound [^{3}H]PDB was separated from free [^{3}H]PDB by adding 1 ml of filtering solution (20 mM Tris-HCl, pH 7.5, 10 mM Mg acetate, 1 mM $CaCl_2$), and filtering the mixture through 2.4-cm Whatman GF/C glass fiber filters by suction. The tubes and filters were washed 5 times with 5 ml of filtering solution. The filters were counted in 10 ml of scintillation cocktail (Scinti-Verse II, Fisher Scientific, CA). Specific binding was calculated as total binding minus nonspecific binding obtained in the presence of 2 μM PMA.

RESULTS

PK-C activity is reduced in Alzheimer particulate samples

The assay of PK-C activity was performed using histone III S as the substrate, PMA/PS as the activator and frontal cortex homogenate as the enzyme source. The PK-C activity recovered in the Alzheimer particulate fraction was about half of that in control samples ($P < 0.01$, Table I and Fig. 1). The PK-C activity in the Alzheimer cytosol fraction seems to be higher than controls, although this difference did not reach statistical significance.

PK-C level is lower in Alzheimer samples

One way to quantify PK-C is to use a radioactive activator of PK-C such as [^{3}H]PDB. Because the interaction is stoichiometric, by counting the radioactivity bound to the enzyme, the quantity of PK-C can be determined. [^{3}H]PDB binding again showed that PK-C in the Alzheimer particulate fraction was about half of that in control samples ($P < 0.01$), although the difference in the cytosolic PK-C between Alzheimer and control was not significant (Table II and Fig. 2). Because two-thirds of the total protein was particulate in both Alzheimer and control, the results of Table II imply lower total [^{3}H]PDB binding in Alzheimer (0.64 pmol/mg) than in control (1.19 pmol/mg) total brain homogenate.

Thus, Alzheimer particulate fraction contains fewer PK-C molecules consistent with the lower PK-C activity raising the issue of whether there are specific PK-C substrates which are affected by this reduced PK-C activity. As we previously demonstrated under our experimental conditions, only the cytosolic fraction contains detectable PK-C dependent phosphoproteins[54]. We therefore studied the phosphorylation of the major endogenous PK-C substrate, a M_r 86,000 protein (P86) found in the cytosol.

P86 phosphorylation is reduced in Alzheimer cytosol

To determine the appropriate incubation time for the phosphorylation reaction, the time course of the incorporation of ^{32}P from [^{32}P]ATP into a major protein kinase C substrate, P86, and into total proteins was followed using cytosol fractions under conditions activating PK-C. Under the conditions employed, the phosphorylation reaction was rather slow (Fig.

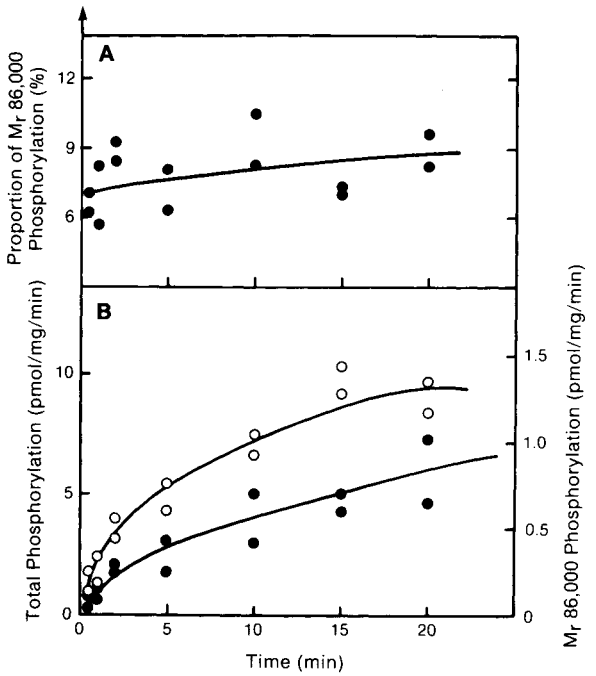

Fig. 3. Time course of the phosphorylation under a PK-C-activating condition. Cytoplasmic proteins (6.5 μg) from human frontal cortex were incubated in 25 μl of medium containing [^{32}P]ATP in the presence of PK-C activators. After various incubation times, the phosphorylation reaction was stopped by adding 6.5 μl 5 × SDS sample buffer, and total protein separated by SDS gel. The M_r 86,000 protein (P86), a PK-C substrate, band was localized by autoradiogram, and the radioactivity recovered in the band as well as that in the total lane (total protein) was determined by liquid scintillation counting. Radioactivity in small proteins which comigrate with tracking dye were excluded from the total radioactivity. The proportion of the P86 phosphorylation to the total protein phosphorylation (A), and the incorporation of radioactivity into the P86 (solid circle) and into the total proteins (open circle) (B) are plotted against incubation time.

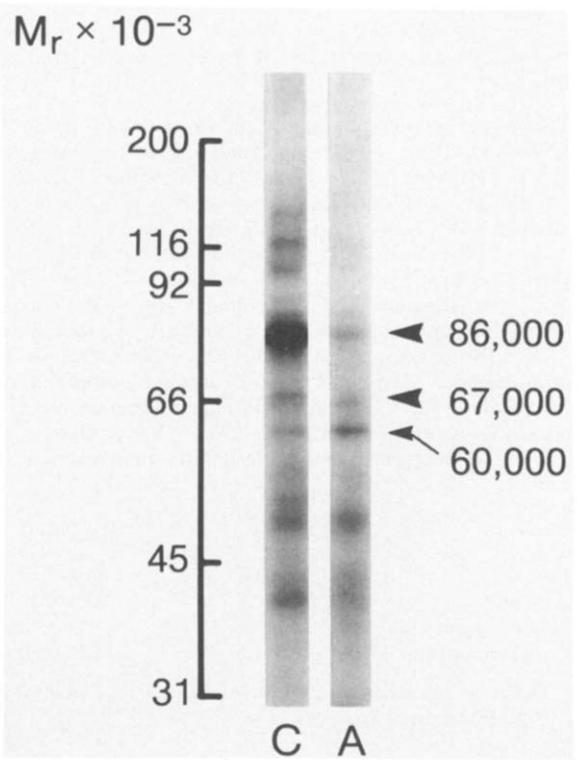

Fig. 4. Phosphorylation of the M_r 86,000 protein (P86), a major PK-C substrate is reduced in Alzheimer cytosol. Cytosolic proteins were phosphorylated under PK-C activating conditions as described under Materials and Methods. Samples were run on SDS gels and autoradiographed. Typical autoradiograms are presented. The same amounts of protein (6.5 μg/lane) were loaded on the gel. C, normal control; A, Alzheimer.

3B) and did not decline significantly up to 15 min. The proportion of the P86 phosphorylation to the total phosphorylation was roughly constant, with a tendency to increase with time (Fig. 3A). For the study described below, we used a 12-min incubation time, which yields substantial (approximately 500 cpm) P86 radioactivity.

Autoradiograms of phosphoproteins, labeled in vitro with [^{32}P]ATP under a condition where PK-C is activated, showed a reduced phosphorylation of certain proteins in the Alzheimer compared to the control sample (Fig. 4). Among the reduced phosphoprotein bands were those of 150,000, 120,000, 100,000, 86,000 and 67,000 with the major reduction in the M_r 86,000 band (P86). The two major bands (M_r 86,000 and 67,000) reported to be PK-C substrates[54] are indicated by arrowheads in Fig. 4. The arrow in Fig. 4 shows a M_r 60,000 protein which has phosphorylation levels elevated in Alzheimer cytosol[53]. The reduced P86 phosphorylation in the cytosol of Alzheimer patients does not seem to result from the translocation of PK-C from the cytosol to the particulate fraction because it was also observed when assayed in the total homogenate. Reduced stimulation by PK-C activators of the P86 phosphorylation was observed in the cytosol fraction from Alzheimer patients when compared with normal controls or non-Alzheimer dementia patients (Table III). Consequently, Alzheimer cases show markedly reduced levels of P86 phosphorylation under PK-C activating conditions when compared to control ($P < 0.002$; Table III; Fig. 5). It seems that this change is rather specific for Alzheimer's disease, as the proportion of P86 phosphorylation in demented patients without

TABLE III

Reduced phosphorylation of the M_r 86,000 protein in Alzheimer cytosol

The proportion of P86 phosphorylation assayed in the presence of PMA/PS (P86), and the stimulation of P86 phosphorylation by PMA/PS [Stimulation = (with PMA/PS)/(without PMA/PS)] are tabulated for 3 different categories of samples. Values are means ± S.D. Controls were from 7 individuals diagnosed to be normal. Their mean (± S.D.) age of death was 67.9 ± 7.1 years, and their mean (± S.D.) postmortem time was 16.2 ± 15.5 h. Alzheimer samples contain 13 cases. Their mean (± S.D.) age of death was 75.5 ± 7.6 years, and their mean (± S.D.) postmortem time was 7.1 ± 6.1 h. Non-Alzheimer dementia samples are 3 Pick's cases, 2 Parkinson's cases and one each of Binswanger's disease, multi-infarct dementia, and hippocampal sclerosis. Their mean (± S.D.) age of death was 77.3 ± 7.5 years and their mean (± S.D.) postmortem time was 5.2 ± 3.8 h.

	P86 (%)	*Stimulation*
Control	13.6 ± 4.5	1.74 ± 0.18
Alzheimer	5.8 ± 2.9*	1.05 ± 0.19*
Non-Alzheimer (demented)	10.5 ± 4.5	1.91 ± 0.56

* Different from control or non-Alzheimer dementia, $P < 0.002$ by unpaired Student's *t*-test.

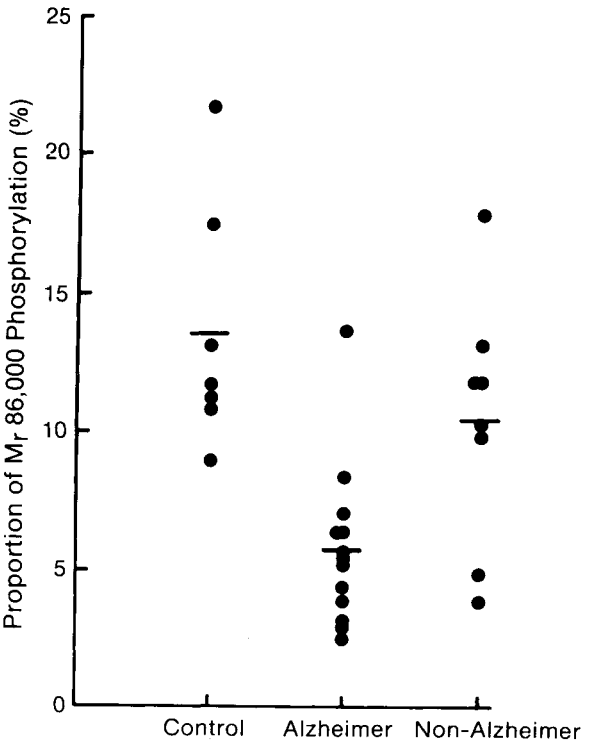

Fig. 5. Scattergram of the M_r 86,000 protein (P86) phosphorylation. The proportion of the P86 phosphorylation relative to the total phosphorylation, excluding the phosphorylation of small peptides which migrate with tracking dye, under PK-C activating conditions is plotted in three categories: control; Alzheimer; non-Alzheimer dementia. The control group is comprised of non-demented patients. All the Alzheimer patients had neuritic plaques and/or neurofibrillary tangles. Demented patients without any plaques or tangles were classified as non-Alzheimer dementia. Student's *t*-test shows the Alzheimer group is significantly different from the control ($P < 0.002$), although the non-Alzheimer dementia group is not significantly different from the control.

Alzheimer's disease is not statistically different from that of the control group (Fig. 5).

Several possible factors which may affect P86 phosphorylation were taken into consideration. First, there may be gender differences in the amount of kinase or substrate and, therefore, the difference in the sex ratios among our 3 groups of patients might affect interpretation of results. Under our experimental conditions, however, there were no differences in the P86 phosphorylation in male or female samples. Second, the age of the patients from whom the autopsy brains were taken may affect the P86 phosphorylation. This point needs specific clarification because the average age of the control group was less than that of the Alzheimer group in this specific experiment (not in other experiments). Still, the plot of the P86 phosphorylation against age does not show any age-dependent change (Fig. 6).

The possibility that the P86 phosphorylation is far more vulnerable to postmortem changes in the Alzheimer sample was studied by plotting P86 phosphorylation against postmortem time. As shown in Fig,. 7, during the course of postmortem delay, the P86 phosphorylation does not appear to decline faster in Alzheimer than in control samples.

DISCUSSION

Alzheimer's brain tissue displays reduced levels of PK-C in the particulate fraction as compared to the control. These reduced PK-C levels were demonstrated either by the PMA/PS-dependent phosphorylation of exogenously added histone or by the [^{3}H]-PDB binding. It is interesting to note that PK-C levels in Alzheimer cytosol detected by these methods are somehow higher than control, although the difference is not statistically different. Consequently, the total PMA/PS-dependent phosphorylation of histone was not lower in Alzheimer homogenate than control homogenate. This might result from the altered compartmentalization of PK-C (a shift from

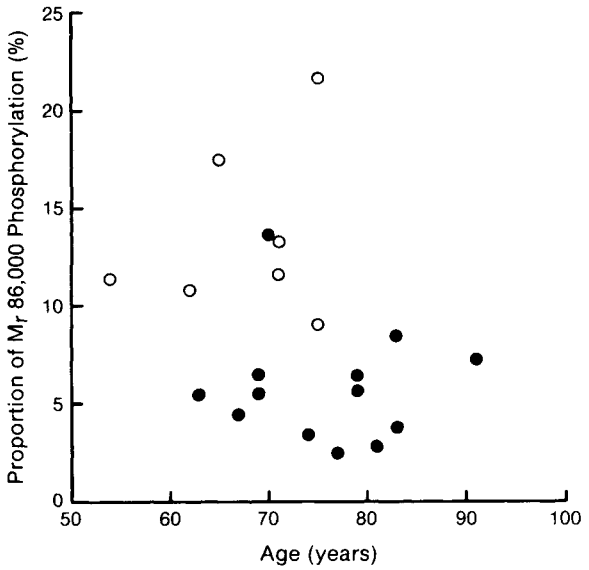

Fig. 6. M_r 86,000 protein (P86) phosphorylation as a function of age. The proportion of the P86 phosphorylation to the total protein phosphorylation excluding the phosphorylation of small peptides was plotted against the age of the patients. Control groups are presented with open circles and Alzheimer groups with solid circles. Note that there is no age-dependent change.

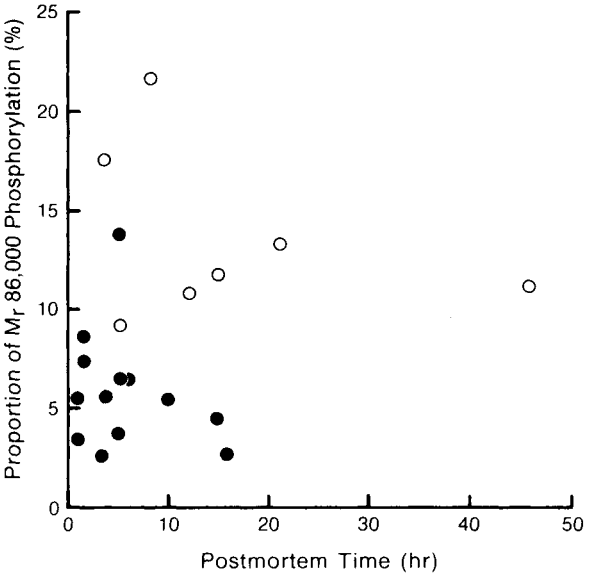

Fig. 7. M_r 86,000 protein (P86) phosphorylation as a function of postmortem time. Same as Fig. 4, except that the abscissa is the postmortem time. No statistically significant regression lines were obtained.

membrane-bound to free) in Alzheimer brain. Because the activation of PK-C shifts free PK-C to the membrane-bound form[30], and because it is probable that neurons in Alzheimer patients fire less frequently than in healthy people and, thus, activate less PK-C, less PK-C in the Alzheimer particulate fraction than in control is not surprising. However, this explanation cannot account for results of the [^{3}H]PDB binding study, where the total [^{3}H]PDB binding activity was reduced in Alzheimer homogenate. PK-C is composed of several isoforms, all of which bind phorbol esters and which are differentially activated by slightly different conditions[26,28,29,56]. This is especially important because the different isoenzymes of PK-C have slightly different calcium affinities which may result in preferential activation of one or another isoenzyme in a particular compartment. Thus, one possible explanation of our results is that a certain isoform(s) of PK-C is reduced in Alzheimer brain, which was detected preferentially by [^{3}H]PDB binding study, while another isoform(s) which was detected preferentially by our histone phosphorylation assays changes its compartmental localization in the brain tissue of Alzheimer patients. Further, the variable cell type and region specific distribution of these PK-C isoforms[38] may help account for the selective cell loss. Studies with isoform specific probes are clearly required. In addition to multiple PK-C isoforms, the interpretation of our PK-C data is further complicated by the heterogeneous subcellular distribution of the enzyme, a distribution consistent with multiple functions. For example, PK-C has been localized in the presynaptic terminals of certain types of neurons[61,62]. In the hippocampus, the effect of muscarinic agonists can be imitated by phorbol ester, suggesting the involvement of PK-C in the intracellular signaling mechanism triggered by the muscarinic acetylcholine receptor[34]. Acetylcholine as well as serotonin, vasopressin, and substance P have been shown to stimulate PK-C[43]. Because acetylcholine, serotonin, and substance P are reduced in brain tissues from Alzheimer patients possibly because of neuronal degeneration, the reduced PK-C levels may be related to the selective neuronal death occurring in the brain of the Alzheimer patient.

PK-C has also been localized in the neuronal nucleus[19,61], suggesting that it is involved in regulation of gene expression. This putative function of PK-C on the regulation of gene expression has been well substantiated at the molecular level[2]. If reduced particulate fraction PK-C levels reflect a reduction in nuclear PK-C associated with the regulation of gene ex-

pression required for neuronal survival, our finding may be related to the cause of neuronal degeneration in Alzheimer's disease. This possibility demands further investigation.

The results of in vitro phosphorylation of endogenous substrates by endogenous PK-C were rather surprising. Although there is no hint of reduced PK-C in the cytosol fraction of Alzheimer brain samples, it displays reduced levels of P86 phosphorylation under PK-C activating conditions. Reduced levels of the P86 phosphorylation were not detected in brain tissues of demented patients without signs of Alzheimer's disease, i.e. without neurofibrillary tangles or neuritic plaques (Table III and Fig. 5). Therefore, reduced P86 phosphorylation does not appear to be related to dementia per se. Furthermore, it is notable that 3 of the non-Alzheimer-demented cases used in the current study had Pick's disease, with severe neuronal deterioration in the frontal cortex. All 3 Pick's cases demonstrated levels of P86 phosphorylation (13.2, 10.3 and 17.9%) comparable to normal control cases, negating the possibility that reduced P86 phosphorylation in Alzheimer's disease is the result of nonspecific neuronal loss common to these diseases.

We do not yet have a molecular explanation for the reduced levels of P86 phosphorylation in the Alzheimer brain cytosol. That is, which component of the Alzheimer sample, enzyme (kinase and phosphatase) or substrate, is responsible for the decreased P86 phosphorylation? PK-C activity measured, using histone as exogenous substrate, did not reveal any reduced PK-C activity in Alzheimer cytosol (in contrast to particulate fraction). However, it is possible that a particular isoform of PK-C is missing in Alzheimer cytosol. Further study, utilizing specific molecular probes, will be necessary to clarify this point.

PK-C has been demonstrated to be masked or inactivated in the presence of Ca^{2+}/calmodulin[1,54,63] and certain Ca^{2+} binding proteins[36,55]. It is possible that increased levels of Ca^{2+}/calmodulin or kinase inhibitors in Alzheimer brain may cause the decreased P86 phosphorylation. Because the calcium metabolism of skin fibroblasts from Alzheimer donors is altered[46–48], it is not unreasonable to speculate that neurons in the Alzheimer patient may have altered calcium levels. It has been reported that certain tangle-bearing neurons have increased Ca^{2+} content[18]. We addressed the question of possible inhibitory molecules in Alzheimer samples by assaying in vitro phosphorylation of a mixture of Alzheimer and control samples. The degree of P86 phosphorylation in the mixture should be smaller than the sum of P86 phosphorylation in each sample if elevated levels of inhibitors are present in the Alzheimer tissue. However, the extent of the P86 phosphorylation in the mixture was the simple sum of the respective phosphorylations. This result supports neither the presence of elevated inhibitor levels nor increased phosphatase activity in the Alzheimer samples.

An increased level of protease activity which degraded proteins during homogenization could explain the reduced levels of P86 phosphorylation. We employed a cocktail of protease inhibitors to prevent proteolytic degradation during the course of the preparation of brain extracts. We were unable to detect proteolytic degradation by studying Coomassie brilliant blue staining of the Alzheimer samples, although the method may not be sensitive enough and P86 may be exceptionally sensitive to proteolytic attack. Furthermore, we cannot exclude the possibility that more proteolytic degradation of P86 occurs during the postmortem delay in the Alzheimer brains.

PK-C plays a pivotal role in the regulation of gene expression[2]. As demonstrated by our [^{3}H]PDB binding study, PK-C levels are lower in brain tissues from Alzheimer patients. Thus, it is possible that the expression of certain genes, including P86, is reduced in the brain of Alzheimer patients. This might be an example of double regulation of a single protein by PK-C, the first regulation at the transcription level and the second one at the post-translational level. This idea must await further experimental support.

Whatever the reason for the reduced levels of P86 phosphorylation and PK-C, because the phosphorylation of selected proteins by PK-C is involved in the survival and maintenance of neurons[24,25,39], our finding may provide a new approach to the study of molecular deficits in Alzheimer's disease.

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